University of Alberta

Intestinal Ganglioside Absorption and Bowel Protective Mechanisms of Gangliosides in an Infant Model of Necrotizing Enterocolitis

By

Kareena Leanne Schnabl

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Experimental Medicine

Department of Medicine

Edmonton, Alberta

Fall 2007

Library and Archives Canada

Published Heritage Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-33058-6
Our file Notre référence
ISBN: 978-0-494-33058-6

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

ABSTRACT

Necrotizing enterocolitis (NEC) is an inflammatory bowel disease of neonates. Hypoxia-ischemia, infection and enteral feeding are risk factors while feeding human milk is protective. The role of vasoactive and inflammatory mediators in the pathogenesis of NEC remains elusive due to limitations in models. Ganglioside GD3 is a glycosphingolipid abundant in colostrum, developing tissues and some tumors. Research demonstrates anti-inflammatory properties of dietary GD3 in rat intestine during infection. The mechanism and efficiency of ganglioside absorption by human intestine has not been investigated.

Models to study GD3 absorption by human intestinal cells and ganglioside roles in inflammatory responses of infant bowel to infection and hypoxia were developed. It was hypothesized that 1) enterocyte GD3 uptake would be time- and concentration-dependent with uptake efficiency and fate influenced by route of delivery and 2) that gangliosides would protect infant bowel by restoring vasoactive and inflammatory mediator balance during infection and hypoxia.

CaCo-2 cells were exposed to GD3 on the apical or basolateral membrane (BLM) side for 6, 24 and 48 h. GD3 uptake, retention, transfer and metabolism were determined. Cultured infant bowel was exposed to *E. coli* lipopolysaccharide (LPS) and hypoxia with or without a period of pre-exposure to ganglioside. Infant bowel necrosis and production of nitric oxide (NO), endothelin-1 (ET-1), serotonin, eicosanoids, hydrogen peroxide (H_20_2) and pro-inflammatory cytokines were measured.

GD3 uptake across the apical and BLM was time- and concentration-dependent and reached a plateau.GD3 uptake across the BLM was more efficient than apical delivery.

Apical GD3 was metabolized with some cell retention and transfer while basolateral GD3 was completely metabolized. Ganglioside pre-exposure reduced bowel necrosis and endothelin-1 production in response to LPS. Gangliosides also suppressed infant bowel production of NO, LTB_4, PGE_2, H_2O_2, IL-1β, IL-6 and IL-8 in response to LPS and hypoxia.

This study demonstrates efficient GD3 uptake by enterocytes and suggests route of delivery influence on ganglioside uptake efficiency and fate. Moreover, this study shows that gangliosides protect infant bowel by restoring vasoactive and inflammatory mediator balance during infection and hypoxia. These findings support potential use of ganglioside-containing enteral or parenteral products to prevent inflammatory bowel disorders.

ACKNOWLEDGEMENTS

My PhD graduate student experience at the University of Alberta was incredible and I am very fortunate to have had the opportunity to work with such a wonderful group of fun, enthusiastic and brilliant individuals.

I would especially like to thank my graduate supervisor, Tom Clandinin and my supervisory committee for their guidance, support and enthusiasm throughout my program. They were there for me through the moments of joy and frustration and have taught me the foundation of everything I need to know to become a successful professional in nutrition and medicine. I look back now and truly appreciate the challenge I was given by Tom to work independently. His openness to my ideas and trust in giving me the responsibility of running a new instrument and making important decisions encouraged me to work above and beyond my potential and as a result I have accomplished more than I ever imagined. Alan Thomson taught me the value of self-reflection and it is through our discussions that I developed as an individual and discovered who I am and what I am capable of achieving. I would also like to thank John Van Aerde, Bodil Larsen, Gordon Lees, Mark Evans, the pediatric nurses and the parents/infants that made the clinical portion of my thesis project possible and a remarkable success. It is my enjoyable experiences working together with the hospital that guided me onto my career path. It is with great excitement that I move forward into a postdoctoral fellowship with opportunities to learn new analytical methods and help patients. Tom's excitement for research is contagious so I can't help but be passionate about continuing clinical research.

Everyone within the Department's of Medicine and Agriculture, Forestry and Nutrition offered assistance during my program and I greatly appreciated it. I would especially like to thank Dick Jones and Sharon Campbell, who made my interdisciplinary program possible and helped out with all the administrative details and personal issues that came about along the way. I very much enjoyed working with the graduate education committee as well as the teaching experiences with Catherine Field, Vera Mazurak, Spencer Proctor and Stefanie Alloway. Special thanks goes out to Dr. Goh, Sue Goruk, Miyoung Suh, Eek Park, Trish Biondo, Megan Ruth, Adrienne Decorby, Marjorie Larcelet, Julie, Laurie Drowsdowski and John Walker who went out of their way to teach me lab techniques or help trouble-shoot experiments.

I would also like to thank my family, friends, the Clandinin clan, my summer students, my soccer team and all the graduate students for their advice and continuous support of my daily life situations and future ambitions. My parents, boy friend Paul, brother Brad, Zahra, Pauline, Rabban, Anchalee, Danielle, Meghan, Ellen, Jen, Mich, David, Amid, Vanessa L, Vanessa H, Nancy, Olga, Sara, Kelly, Qun, Ben and Andrea especially brightened my days with their kind words, glowing smiles and endless laughter.

Finally I would like to acknowledge the financial support from the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada. The travel grants from the University of Alberta and other organizations also supported my research by giving me the opportunity to present and network at conferences. The source of ganglioside used in my study was provided by Fonterra, New Zealand.

TABLE OF CONTENTS

Table of Contents

List of Tables

List of Figures or Illustrations

List of Abbreviations

CHAPTER 1 THESIS OUTLINE AND LITERATURE REVIEW

1.0 Thesis Outline 1

Gangliosides 1

1.1 Classification of Gangliosides as Glycosphingolipids 1

1.2 Chemical Structure of the Ganglioside 2

1.3 Nomenclature of Glycosphingolipids 3

1.4 Physical Properties of Gangliosides 4

1.5 Food Sources and Dietary Intake 5

1.6 Distribution and Composition of Gangliosides 6

1.6.1 Intestine 7

1.6.2 Neural Tissues 8

1.6.3 Immune Cells 8

1.6.4 Milk 9

1.6.5 Tumors and Cancer Cells 10

1.7 Ganglioside Metabolism 10

1.7.1 Digestion, Absorption and Transport 10

1.7.2 Biosynthesis of Gangliosides 12

1.7.3 Degradation of Gangliosides 13

1.7.4 Ganglioside Turnover and Recycling 15

1.8 Biological Functions of Gangliosides 16

1.8.1 Cell Recognition, Adhesion and Migration 16

1.8.2 Intestine Barrier Function, Signal Transduction and Pathogen Entry into Microdomains (Lipid Rafts and Caveolae) 17

1.8.3 Cell Proliferation, Differentiation and Apoptosis 18

1.8.4 Ganglioside Function in Calcium Homeostasis and Signaling 19

1.8.5 Immune System Development and Function 20

1.9 Pathological and Pharmacological Function of Gangliosides in Disease 21

1.9.1 Inherited Sphingolipid Disorders 21

1.9.2 Neurodegenerative Diseases and Neuron Repair 23

1.9.3 Cancer 24

1.9.4 Inflammatory Diseases and Infection 25

Necrotizing Enterocolitis 25

1.10 Definition, Epidemiology and Prognosis 25

1.11 Diagnosis 26

1.11.1 Clinical Signs and Symptoms 26

1.11.2 Pathological Findings 27

1.11.3 Diagnostic Methods 28

1.12 Pathogenesis 29

1.12.1 Pre-Maturity 30

1.12.1.1 Immature Intestinal Motility, Digestion, Barrier Function 30

1.12.1.2 Immature Intestinal Immunity 33

1.12.2 Hypoxia-Ischemia 34

1.12.3 Formula Feeding 36

1.12.4 Abnormal Bacteria Colonization and Infection 36

1.12.5 Genetics 39

1.12.6 Vasoactive and Inflammatory Mediators 40

1.12.6.1 Nitric Oxide 41

1.12.6.2 Endothelin-1 42

1.12.6.3 Serotonin 43

1.12.6.4 Platelet Activating Factor 43

1.12.6.5 Eicosanoids 44

1.12.6.6 Cytokines 46

1.12.6.7 Reactive Oxygen Species 52

1.13 Necrotizing Enterocolitis Models 53

1.14 Symptomatic Treatment and Surgery 54

1.15 Prevention 55

1.15.1 Human Milk 56

1.15.2 Trophic Feeds 56

1.15.3 Antenatal Glucocorticoids 57

1.15.4 Enteral Antibiotics 57

1.15.5 Standardized Feeding Regimens 57

1.15.6 Fluid Restriction 58

1.15.7 Probiotics 58

1.15.8 Prebiotics 59

1.15.9 Postbiotics 59

1.15.10 Arginine Supplementation 60

1.15.11 Free Radical Scavengers (Anti-Oxidants) 60

1.15.12 Acidification of Gastric Feeds 60

1.15.13 Polyunsaturated Fatty Acids 61

1.15.14 Oral Immunoglobulins 61

1.15.15 Epidermal Growth Factor 61

1.15.16 Erythropoietin 62

1.15.17 Other Experimental Agents 62

1.16 References 63

CHAPTER 2 RESEARCH PLAN 83

2.1 Introduction and Rationale 83

2.2 Objective and Specific Aims 84

2.3 Hypotheses 85

CHAPTER 3 CHANGE IN GANGLIOSIDE COMPOSITION DURING HUMAN COLON CANCER CELL DIFFERENTIATION

3.1 Introduction 87

3.2 Materials and Methods 89

3.2.1 Cell Culture 89

3.2.2 Determination of Cell Protein 90

3.2.3 Assessment of Cell Differentiation Markers 90

3.2.4 Ganglioside Extraction 90

3.2.5 Analysis of Total and Individual Ganglioside Content 91

3.2.6 Statistical Analysis 92

3.3 Results 92

3.3.1 Ganglioside content of undifferentiated/differentiated CaCo-2 cells 92

3.3.2 Individual ganglioside composition of undifferentiated & differentiated human colon cancer CaCo-2 cells 94

3.3.3 Individual ganglioside composition in undifferentiated and differentiated human colon cancer CaCo-2 cells 94

3.4 Discussion 96

3.5 References 99

CHAPTER 4 GANGLIOSIDE GD3 ABSORPTION BY HUMAN INTESTINAL CACO-2 CELLS

4.1 Introduction 103

4.2 Experimental Procedures 106

4.2.1 Materials 106

4.2.2 Cell Culture 107

4.2.3 Isolation and Purification of Gangliosides 107

4.2.4 Intestine Cell Sensitivity to Gangliosides 108

4.2.5 Ganglioside Uptake by Human Intestine Cells 108

4.2.6 Determination of Cell Protein 109

4.2.7 Ganglioside Analysis 110

4.2.8 Calculating Uptake, Retention, Transfer and Metabolism of GD3 110

4.2.9 Statistical Analysis 111

4.3 Results 111

4.3.1 Sensitivity of human intestinal CaCo-2 cells to ganglioside GD3 111

4.3.2 Time-dependent uptake of GD3 by human intestinal CaCo-2 cells 112

4.3.3 Concentration-dependent uptake of GD3 by human intestinal CaCo-2 cells 113

4.3.4 Fate of ganglioside GD3 taken up by human intestinal CaCo-2 cells 114

4.4 Discussion 116

4.5 References 123

CHAPTER 5 GANGLIOSIDES PROTECT BOWEL IN AN INFANT MODEL OF NECROTIZING ENTEROCOLITIS BY SUPPRESSING VASOACTIVE AND PRO-INFLAMMATORY SIGNALS OF INFECTION AND HYPOXIA

5.1 Introduction 128

5.2 Methods 129

5.2.1 Subjects and Bowel Tissue 129

5.2.2 Cell and Tissue Culture 130

5.2.3 Oxygen Consumption 131

5.2.4 Extraction, Purification and Analysis of Gangliosides 131

5.2.5 Infant Bowel Model of Necrotizing Enterocolitis 132

5.2.6 Tissue Viability and Integrity 133

5.2.7 Vasoactive Mediator Production 133

5.2.8 Eicosanoid Extraction 134

5.2.9 Eicosanoid Analysis 134

5.2.10 Hydrogen Peroxide 135

5.2.11 Cytokine Production 135

5.2.12 Statistical Analysis 136

5.3 Results 136

5.3.1 Gangliosides improve bowel survival during infection in an infant model of necrotizing enterocolitis 136

5.3.2 Gangliosides inhibit nitric oxide release from infant bowel during infection and hypoxia 139

5.3.3 Gangliosides reduce infant bowel production of vasoconstrictors, endothelin-1 and serotonin, during infection and hypoxia 140

5.3.4 Gangliosides inhibit eicosanoid and hydrogen peroxide production by infant bowel during infection and hypoxia 140

5.3.5 Gangliosides inhibit pro-inflammatory cytokine production in infant bowel during infection and hypoxia 143

5.4 Discussion 146

5.5 References 151

CHAPTER 6 THESIS CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Thesis Conclusions 155

6.2 Thesis Summary 159

6.3 Significance of Thesis Research Findings 160

6.4 Future Directions 161

6.4.1 Mechanism of Action 162

6.4.2 Pharmacokinetics/Pharmaceutics 163

6.4.3 Broader Clinical Applications 163

6.4.3.1 Atherosclerosis and Prevention of Myocardial Infarction/Stroke 164

6.4.3.2 Prevention of Ischemic Injury in Organ Transplantation 164

6.4.3.3 Systemic Sclerosis 165

6.4.3.4 Shock in Trauma, Burn or Critically Ill Patients 165

6.5 References 166

List of Tables

Table 1-1	Nomenclature Systems for Gangliosides
Table 1-2	Sphingolipids in Food
Table 1-3	Ganglioside Composition of Rat Small Intestinal Mucosa
Table 1-4	Summary of Major Sphingolipidoses
Table 1-5	Bell's Staging Criteria for Necrotizing Enterocolitis
Table 1-6	Ischemic Events Associated with Necrotizing Enterocolitis
Table 1-7	Eicosanoid Synthesis and Actions
Table 1-8	Strategies to Prevent Necrotizing Enterocolitis
Table 4-1	Uptake, retention, transfer and metabolism of apical ganglioside GD3 by human intestine CaCo-2 cells over time
Table 4-2	Uptake, retention, transfer and metabolism of basolateral ganglioside GD3 by human intestine CaCo-2 cells over time
Table 5-1	Endothelin-1 and serotonin production by infant bowel following exposure to enterotoxic *E. coli* LPS, hypoxia or the combination in the presence and absence of a 10 h period of pre-exposure to ganglioside

List of Figures and Illustrations

Figure 1-1	Classification of Membrane Lipids
Figure 1-2	Chemical Structure of Gangliosides
Figure 1-3	Ganglioside Synthesis by Membrane-Bound Glycosyltransferases
Figure 1-4	Degradative Pathways for Gangliosides
Figure 1-5	Subcellular Sites of Ganglioside Metabolism and Traffic
Figure 1-6	Pathophysiology of Necrotizing Enterocolitis
Figure 1-7	LPS-Induced Signaling Pathways Leading to NFκβ Activation
Figure 1-8	Metabolic Pathways of Arachidonic Acid and Eicosanoid Production
Figure 1-9	Schematic of Chamber for Growing Intestinal Epithelial Cells on Polycarbonate Membranes
Figure 3-1	Simplified Schematic of Ganglioside Biosynthesis and Degradation
Figure 3-2	Total content of ganglioside-bound N-acetyl neuraminic acid in undifferentiated and differentiated human colon cancer CaCo-2 cells
Figure 3-3	Individual ganglioside composition of undifferentiated and differentiated CaCo-2 cells
Figure 3-4	Ganglioside composition of undifferentiated and differentiated CaCo-2 cells measured by a colorimetric N-acetyl neuraminic acid (NANA) assay and scanning densitometry
Figure 4-0	Mechanisms proposed for ganglioside absorption by intestine and fate of absorbed gangliosides
Figure 4-1	Schematic of intestinal epithelial monolayers grown on trans-well plates

Figure 4-2	Sensitivity of human intestinal CaCo-2 cells to ganglioside GD3
Figure 4-3	Percentage uptake of ganglioside GD3 into human intestinal CaCo-2 cells over time
Figure 4-4	Amount of ganglioside GD3 uptake into human intestinal CaCo-2 cells
Figure 5-1	Development of an infant bowel model for necrotizing enterocolitis
Figure 5-2	Inhibitory effect of gangliosides on infant bowel production of nitric oxide during infection and hypoxia
Figure 5-3	Inhibitory effect of gangliosides on infant bowel production of pro-inflammatory eicosanoids and hydrogen peroxide during infection and hypoxia
Figure 5-4	Infant bowel pro-inflammatory cytokines involved in the pathogenesis of necrotizing enterocolitis and anti-inflammatory effects of gangliosides during infection and hypoxia
Figure 5-5	Pathogenesis of necrotizing enterocolitis and mechanism by which gangliosides protect infant bowel from inflammation and injury

List of Abbreviations

ABC	adenosine triphosphate (ATP) binding cassette
BBM	brush border membrane
BLM	basolateral membrane
CaCo-2	human colon cancer cell line
Cer	ceramide
EGF	epidermal growth factor
EMEM	Earle's minimum essential medium
eNOS	endothelial nitric oxide synthase
Epo	erythropoietin
ET-1	endothelin-1
FBS	fetal bovine serum
Gal	galactose
Gly	glucose
GM-CSF	granulocyte macrophage colony stimulating factor
GPI	glycosylphosphatidylinositol
HDL	high density lipoprotein
IBD	inflammatory bowel disease
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
LDH	lactate dehydrogenase

LDL	low density lipoprotein
LPS	lipopolysaccharide
LT	leukotriene
MDR	multi-drug resistance
MHC	major histocompatability complex
NANA	N-acetyl neuraminic acid
NEC	necrotizing enterocolitis
NFκβ	nuclear factor kappa beta
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOD2	nucleotide-binding oligomerization domain 2
PAF	platelet activating factor
PBS	phosphate buffered saline
PG	prostaglandin
PMN	polymorphonuclear
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
SA	sialic acid
SAT	sialyl transferase
TGF	tumor growth factor
TNF	tumor necrosis factor
VLBW	very low birth weight
VLDL	very low density lipoprotein

CHAPTER 1 THESIS OUTLINE AND LITERATURE REVIEW

1.0 Outline of Thesis

Chapter 1 provides an extensive review of the literature on the nutrient and disease of research focus in this thesis, the ganglioside and necrotizing enterocolitis, respectively. The rationale for undertaking research in the area of ganglioside absorption and necrotizing enterocolitis will be discussed in Chapter 2 along with thesis research objectives, aims and hypotheses. The ganglioside composition of undifferentiated and differentiated human colon cancer CaCo-2 cells is determined in Chapter 3 to determine whether differentiated CaCo-2 cells are a suitable model for infant gut. In Chapter 4, ganglioside GD3 absorption is studied using polarized monolayers of human intestinal CaCo-2 cells. An infant bowel model for necrotizing enterocolitis is developed in Chapter 5 and used to investigate the regulatory role of gangliosides in the inflammatory responses of infant bowel to LPS exposure and hypoxia. Finally, Chapter 6 summarizes the significant thesis findings and suggests potential future directions in ganglioside research.

GANGLIOSIDES

1.1 Classification of Gangliosides as Glycosphingolipids

Glycosphingolipids are the most structurally diverse and complex group of amphipathic membrane lipids found in species ranging from bacteria to humans[1,2]. Glycosphingolipids are glycosylated ceramides composed of sphingosine and a fatty acid that carries one or more carbohydrate residues[1]. Glycosphingolipids are divided into two major categories, neutral and acidic glycosphingolipids, depending on the nature of their hydrophilic oligosaccharide chain component[1]. Neutral glycosphingolipids consist of a ceramide with one or more uncharged sugars like glucosyl- or galactosylceramide while acidic glycosphingolipids consist of a ceramide with ionized functional groups or one or more charged sugar residues[1,2]. The five subclasses of acidic glycosphingolipids are urono-, sulfo-, phospho-, phosphono- and sialoglycosphingolipids (gangliosides)[3]. Figure 1-1 summarizes the membrane lipid classification scheme for gangliosides.

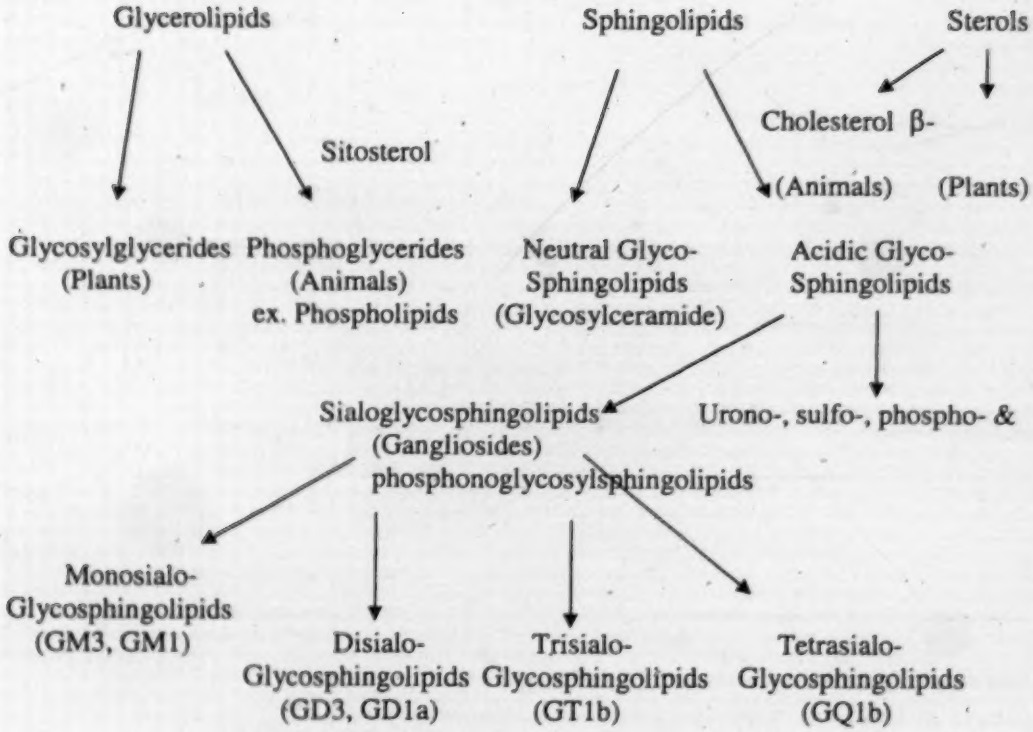


Figure 1-1 Classification of Membrane Lipids

1.2 Chemical Structure of the Ganglioside

Gangliosides are complex glycosphingolipids, which consist of an aliphatic long chain base, a sphingosine attached to a fatty acid by an amide bond, and one or more sugar residues with one or more sialic acid (also called neuraminic acid) moieties attached[4]. The chemical structure of the ganglioside is displayed in Figure 1-2. Neuraminic acid can be substituted with either acetyl- or glycolyl- groups[4]. These two different types of neuraminic acid are species-dependent. For example, N-acetyl neuraminic acid (NANA) is present in humans while rats have both acetyl- and glycosyl- neuraminic acids[5].

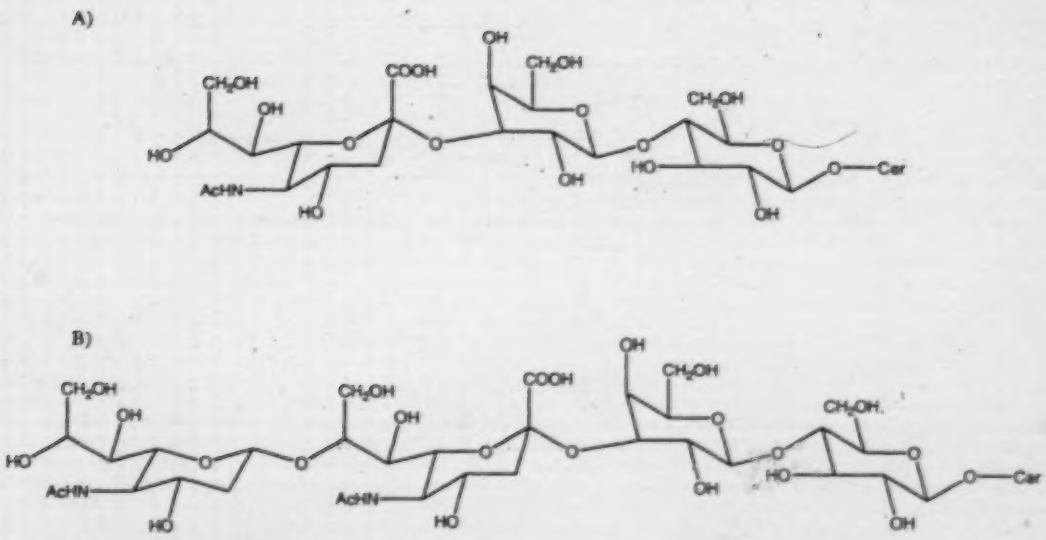


Figure 1-2 Chemical Structure of Gangliosides A) GM3 and B) GD3

1.3 Nomenclature of Glycosphingolipids

There are greater than 400 different structures of glycosphingolipids[1]. The *Nomenclature Commission of the International Union of Biochemistry* abbreviates glycosphingolipid sugar residues with three-letter symbols and ceramide with "Cer"[4]. For example, the neutral glycosphingolipid galactosylceramide is abbreviated GalCer. Diversity in the specific number and pattern of sugars in the core structure of the oligosaccharide chain led to adoption of a nomenclature system for complex glycosphingolipids that identifies the number of sugar units in the chain with a subscript number (mono- (one sugar), di- (two sugars), tri- (three sugars), tetra- (four sugars) etc.), and the sugar pattern with a root name (ganglio-, lacto-, neolacto-, globo- etc.)[3]. In ganglioside nomenclature, roman numerals indicate the position of the monosaccharide residue counting from the ceramide end that sialic acid is attached to while an Arabic superscript indicates the position in the sugar that the sialic acid is attached to[4]. For example, II^3-N-acetylneuraminosylgangliolactosylceramide is II^3-NeuAc-LacCer (GM3) and II^3-N-acetylneuraminosyldigangliolactosylceramide is II^3-$(NeuAc)_2$-LacCer (GD3) (Table 1-1). Svennerholm nomenclature of gangliosides has been commonly used due to its simplicity[4]. The capital letter "G" stands for ganglioside[4]. The capital

letters M, D, T, Q or P that follow correspond to the number of sialic acid residues (mono-, di- tri- etc.), the number that follows identifies the number of sugar residues in the oligosaccharide chain (1 = 4 sugars, 2 = 3 sugars, 3 = 2 sugars etc.) and the small letter subscripts a, b and c represent the biosynthetic pathway of the ganglioside (Table 1-1)[4]. Therefore, GM3 is a monosialoganglioside with two sugars while GD3 is a disialoganglioside with two sugars. GD1a corresponds to a disialoganglioside having four sugar residues different from GD1b synthesized in the b-series pathway. The Svennerholm nomenclature system for gangliosides is shown in Table 1-1[3]. In my thesis, I will use the Svennerholm nomenclature system recommendations.

Table 1-1 Nomenclature Systems for Gangliosides

Svennerholm Code Name	International Union Biochemistry
A Series	
GM3	II^3NeuAcLacCer
GM2	II^3NeuAcGalNAcLacCer
GM1	II^3NeuAcGalGalNAcLacCer
GD1a	IV^3NeuAcII^3NeuAcGalGalNAcLacCer
B Series	
GD3	$II^{3(}$NeuAc$)_2$LacCer
GD1b	$II^{3(}$NeuAc$)_2$GalGalNAcLacCer
GT1b	$II^{3(}$NeuAc$)_2IV^3$NeuAcGalGalNAcLacCer

1.4 Physical Properties of Gangliosides

Gangliosides are insoluble in non-polar solvents and have amphiphilic properties due to a charged hydrophilic portion (oligosaccharide chain with sialic acid) attached to a hydrophobic tail (ceramide)[6]. Owing to their amphiphilic properties, gangliosides form micelles and unilamellar vesicles in aqueous solutions and can spontaneously undergo lateral phase separation in membranes together with cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins into microdomains or clusters (caveolae, lipid rafts)[1,6]. Lipid rafts are membrane microdomains with a different composition to surrounding regions of the membrane[7]. They are enriched in cholesterol, glycosphingolipids, sphingomyelin, phospholipids with long, unsaturated acyl chains,

GPI-linked proteins and some membrane spanning proteins[7]. The lateral organization of glycosphingolipids in the plasma membrane allows the partitioning of proteins, many involved in signal transduction, through selective association with lipid rafts[8]. It has been proposed that the general function of glycosphingolipids in lipid rafts is to provide sites of local enrichment of molecules on both sides of the plasma membrane that need to interact with each other or to be transported to the same place in the cell[7,8].

1.5 Food Sources and Dietary Intake

Sphingolipids are significant components of food[9]. The complex sphingolipids of plants are mainly cerebrosides while foods of mammalian origin have a wide spectrum of complex sphingolipids including sphingomyelin, cerebrosides, globosides and gangliosides[10]. Sphingolipid amounts vary considerably in food with eggs, dairy, meat and fish being the most sphingolipid-enriched food sources[10]. Food consumption data indicates that dairy products and meat are the major source in the human diet and that the average dietary intake for an adult is approximately 0.3-0.4 g of sphingolipid/day[10]. Newborns ingest approximately 400-500 ml of milk per day and a 4 month old baby consumes 550-1000 ml of milk per day[11]. Human milk contains sphingomyelin (39-119 mg/l), glucosylceramide and lactosylceramide (12.5-36 mg/l) and gangliosides (11-16 mg/l), thus suckling babies ingest about 35-170 mg of sphingolipids per day[12,13]. Estimates of the amounts of sphingolipids in several foods are summarized (Table 1-2)[10].

Table 1-2 Sphingolipids in Food

Food Source	Sphingolipid Content (umol/kg)
Dairy Products	
Milk	92
Cheese	1326
Butter	460
Meat, Fish and Alternatives	
Beef	390
Pork	350
Chicken	530
Turkey	390
Fish	130
Eggs	2250
Peanuts	78
Fruits and Vegetables	
Apple	69
Orange	24
Tomato	42
Potato	69

1.6 Distribution and Composition of Gangliosides

The main location of glycosphingolipids is the outer layer of the plasma membrane where the oligosaccharide portions are exposed toward the cell surface and the hydrophobic ceramide moieties are inserted into the surface layer of the membrane[2]. Of the plasma membrane associated glycosphingolipids, 70% are intermixed with other lipids in the membrane and the remaining glycosphingolipids are clustered in lipid rafts or endocytosed micelles[14]. Minor sites of location are the subcellular organelles where glycosphingolipid transport and metabolism occur (endosomes, Golgi apparatus, lysosomes) and biological fluids (milk, blood)[2]. Human plasma contains small amounts of ganglioside as part of lipoproteins, particularly in low density lipoprotein and to a lesser extent in high density lipoprotein[15]. Ganglioside content and composition are species-specific and vary in different tissues, cells and biological fluids suggesting specific roles for gangliosides in distinct physiological processes. Changes in ganglioside content and composition are a general phenomenon characterizing differentiation, development and oncogenic transformation and rely on the balance

between activities of glycosyltransferase enzymes at branch points of ganglioside biosynthesis[16-18]. Dietary intake of ganglioside is also a factor that affects ganglioside content and composition of tissues[19].

1.6.1 Intestine

The microvillus membrane of the rat small intestine contains high amounts of glycosphingolipids including gangliosides[19,20]. Glycosphingolipids account for 20-30% of total lipids in the brush border membrane and gangliosides make up 34% of glycosphingolipids[21]. The dominant ganglioside found in mature rat intestine is GM3 (10.8 nmol NANA per mg protein) and is 5 times higher in the differentiated villus tip (mature cells) than in the undifferentiated crypt cells (proliferative cells)[22]. Ganglioside content and composition of human enterocytes and intestine have not been explored. Table 1-3 displays the ganglioside composition in percentages for the small intestinal mucosa of young rats[19].

Table 1-3 Ganglioside Composition of Rat Small Intestinal Mucosa

Gangliosides	Rat Intestine
GM3	83.5 %
GM2	2 %
GM1	2.7 %
GD3	3.2 %
GD1a	2.3 %
GD1b	1.9 %
GT1b	2.1 %
Total	50 μg/g wet tissue

The intestine undergoes developmental changes in the content and composition of gangliosides. The concentration of gangliosides is lowest in proximal intestine and increases along the length of rat intestine toward the distal segment with regional variation in fatty acid composition[20]. Neonatal rat intestine contains 7 times more GM3 compared to adult intestine[23]. During human embryonic stem cell differentiation, GD3, an abundant ganglioside detected in developing tissues, appears[4,16]. Similar to change occurring in human milk during lactation, the ratio of GM3 to GD3 also increases in the intestinal mucosa during development suggesting a relationship between the presence of individual gangliosides in human milk and immaturity of the gut[4]. Recently, we found that small amounts of dietary ganglioside enriched in GD3 increase membrane content

of GD3 by decreasing GM3 in intestinal mucosa of young rats[19]. Moreover, it was demonstrated that GM3 localizes to the brush border membrane (BBM) and GD3 localizes to the basolateral membrane (BLM)[19]. Considering that the intestine undergoes important transformations in its structure, composition and absorption of nutrients during postnatal development, it is likely that diet induced modifications in membrane lipid and ganglioside content occur that alter protein function in the plasma membranes of intestinal cells.

1.6.2 Neural Tissues

Gangliosides are most abundant in neural tissues and are predominantly localized in neuronal cell membranes of the central nervous system (brain and spinal cord) and peripheral nerves of the retina and autonomic nervous system[19,24,25]. Studies on ganglioside content and composition of enteric nerves have not been undertaken. The major human gangliosides in the CNS are GM1, GD1a, GD1b and GT1b and constitute 10-12% of total lipid content[26]. The total concentration of gangliosides in human peripheral nerves is less than that found in the central nervous system[25]. Based on ganglioside composition studies in human and rat neural tissue, GD3 and GM3 are more abundant in peripheral nerves than the central nervous system[24,25]. The ganglioside content and composition changes during brain development. In the human cerebral cortex, there is a 3 fold increase in ganglioside concentration during postnatal development[27]. Moreover, breast-fed infants have higher levels of brain gangliosides than formula-fed infants at 12 weeks indicating that gangliosides may enhance synaptogenesis and neurodevelopment[5]. Gangliosides in peripheral nerves have also been studied during development. GD3 is the predominant ganglioside during neuroepithelial cell proliferation in the early stage of development in mammalian central and peripheral nerves[27].

1.6.3 Immune Cells

Gangliosides play an important role in differentiation and response reactions of immune cells. The four predominant gangliosides found in peripheral blood lymphocytes are GM3, GD3, GD1a and GT1b[28]. Ganglioside composition shifts have

been associated with lymphocyte differentiation[29]. Furthermore, lymphocytes isolated from different organs are characterized by different amounts of total gangliosides (ratios of lipid-bound sialic acid to protein). Both thymus and leukemic lymphocytes have higher amounts of total gangliosides than peripheral blood lymphocytes[30]. The gangliosides of thymus and leukemic lymphocytes are also characterized as having more GD3 and polar gangliosides and less GM3 gangliosides than peripheral blood lymphocytes[30]. Mitogen stimulation of immune cells has also been shown to profoundly alter ganglioside composition as well as increase ganglioside synthesis. For example, ganglioside synthesis is stimulated in macrophages exposed to endotoxin and activated neutrophils reduce their plasma membrane GM3 content[31,32]. Gangliosides have also been found in neutrophil granules[31].

1.6.4 Milk

Gangliosides in human milk and infant formula are considered to be bioactive components in infant nutrition. Milk gangliosides are part of the membrane fraction of the fat globule[4]. Human and bovine milk vary in the total amount and composition of gangliosides. The total amount of gangliosides in human colostrum and mature human milk is 9.51 mg of lipid bound sialic acid (LBSA) per L and 9.07 mg of LBSA/L, respectively[33]. In contrast, the total amount of ganglioside in mature bovine milk is 2.4 times lower (3.98 mg of LBSA/L)[33]. As a consequence, the ganglioside content in bovine milk based infant formula is also significantly lower compared with human milk. In addition, not only the quantity but also the composition of bovine milk gangliosides differs from those in human milk. The dominant gangliosides in colostrum are GD3 (49%), polar gangliosides (39%) and GM3 (3%)[34]. During lactation, the ratio of GM3 to GD3 increases in human milk (0.2 – 19.82 μmol sialic acid per 100 ml) with decrease occurring in complex polar gangliosides[18,34]. The dominant gangliosides in cow's milk are GD3 (61%), polar gangliosides (23%) and GM3 (3%)[34]. In cow's milk, the GM3 to GD3 ratio increases during the 1st five days of lactation and then drops until the end of lactation[4]. The ganglioside composition of infant formula is similar to cow's milk with higher GD3 (70-80%) and fewer polar gangliosides (4%)[34]. The fatty acid composition of milk gangliosides also differs between human and bovine milk. The

amount of long chain fatty acids (≥ 20 C atoms) is higher in bovine milk gangliosides than human milk gangliosides[33]. Differences in ganglioside content and composition could contribute to the protective effect of human milk against infections and its beneficial effects on gut associated lymphoid tissue development.

1.6.5 Tumors and Cancer Cells

Alteration of ganglioside expression in association with tumor transformation has been established in a large number of tumor tissues and cancer cell lines[35]. The altered expression in cancer cells and tumor tissues comprises over expression of gangliosides found in corresponding normal cells/tissues and expression of gangliosides that are not found in normal tissue but are often expressed during development (i.e. in fetal structures)[35]. Gangliosides GD3, GM3, GD2 and GM2 are generally over-expressed in tumors and have been most extensively explored as tumor antigens[35]. For example, when colon cancer cells differentiate in culture, sialidase, an enzyme that breaks down gangliosides, is down-regulated[17]. Consequently, it would be expected that differentiated colon cancer cells would have more complex, polar ganglioside profiles. It is not known how the amounts of GM3 and GD3 change during colon cancer cell differentiation but it is thought that the less tumorigenic form would have more pro-apoptotic GD3 and less mitotic GM3[17]. Such a ganglioside profile would be characteristic of the ganglioside composition observed in neonatal tissue.

1.7 Ganglioside Metabolism

1.7.1 Digestion, Absorption and Transport

Few studies have investigated the site and mechanism for digestion of glycosphingolipids in the gastrointestinal tract. Based on studies in mice and rats, glycosphingolipid degradation to ceramide, sphingoid bases and free fatty acids is slow and incomplete within all regions of the small intestine and involves the combined action of neutral sphingomyelinase (present in pancreatic juice), alkaline sphingomyelinase (present in bile) and ceramidase[10,36-38]. Alkaline sphingomyelinase and neutral ceramidase are expressed soon after birth and continue to increase and plateau 4 weeks after birth in the small intestine of neonatal rats[39]. The expression of

these enzymes and sphingomyelin digestion is highest in the jejunum and ileum of suckling pigs[40]. Acidic sphingomyelinase is found in lysosomes and secreted in milk while sialidase is present in saliva, gastrointestinal secretions, mucosa and human milk[40]. Intestinal flora is also important for sphingolipid turnover in the lower bowel and helps generate biological compounds that serve as lipid second messengers[10]. Glycosphingolipid digestion is affected by luminal factors such as bile salts, cholesterol and presence of other lipids[41]. Together, these findings suggest that dietary gangliosides may be hydrolyzed by sphingomyelinase, ceramidase and sialidase in the intestinal tract from birth onward and that ganglioside breakdown may be altered by diet.

To date there are no studies that have specifically looked at the mechanism or efficiency of ganglioside uptake and transfer into and across the intestine. It is known that gangliosides form aggregates in aqueous environments such as the stirred water layer and depending on their composition form stable micelles or unilamellar vesicles[6,14]. Incorporation of gangliosides into the plasma membrane has been shown to occur by a time- and concentration-dependent process without chemical alteration[42]. Previous work in our lab shows that ganglioside localization is specific in enterocytes with GM3, the major ganglioside in the intestine, localizing to the brush border membrane and GD3 localizing to the basolateral membrane[19]. Cell culture studies suggest that exogenously administered gangliosides are taken up by a wide range of cells via two different mechanisms: 1) molecules dissociated from micelles, diffuse through the aqueous phase and insert into plasma membranes with or without a protein carrier 2) micelles are taken up by receptor-mediated endocytosis[14]. Serum bovine albumin strongly binds to the oligosaccharide portion of gangliosides and was the first protein thought to play a role in the uptake of gangliosides across the blood brain barrier[43]. More recently, lipoprotein receptors have been identified as a site for sphingolipid uptake from the blood stream. Sphinogomyelin is removed from the blood by tissues through receptor-mediated catabolism of chylomicron and very low density lipoprotein (VLDL) remnants and scavenger receptor B1 mediated transfer into cells[41]. Other candidate proteins for ganglioside uptake and transfer that bind glycosphingolipids include the Siglec family of sialic acid receptors, CD1d present on enterocytes, sterol carrier protein 2 and the multi-drug resistance transporter (MDR1,

also known as P-glycoprotein or ATP-binding cassette (ABCB1))[44-47]. A recent study investigating the uptake of gangliosides by the parasite *Giardia lamblia* demonstrated carrier-independent uptake of liposome gangliosides[42]. The low bioavailability of gangliosides prompted researchers to find alternative methods to enhance delivery of gangliosides. Several researchers have encapsulated gangliosides in liposomes and demonstrated enhanced delivery of liposomal gangliosides to tissues and microorganisms[42]. Liposomes are spherical lipid vesicles formed from aqueous dispersions of amphiphilic molecules such as polar lipids that tend to produce bilayer structures[48]. They are suitable systems for the targeted delivery of a wide variety of substances including nutrients to cells and tissues[48]. Glycolipids are transferred from liposomes to plasma membranes by glycolipid transfer proteins[49].

According to Pagano's vesicle sorting theory[50], absorbed gangliosides have three fates: transport back to the plasma membrane immediately after being endocytosed; endocytosis to the Golgi apparatus for glycosylation to form more complex ganglioside species and transport by the endosome to the lysosome for degradation. Recently, glycolipid transport proteins have been identified in the cytosol that may play a role in non-vesicular transport of glycolipids within cells[51]. It is known that small amounts of ganglioside are able to move across the intestine to the blood as gangliosides have been found in serum lipoproteins, platelets and bound to albumin and leukocytes in the blood[10]. Animal feeding studies in our lab indicate localization of gangliosides in lipid rafts and transport out to plasma[19]. This route would suggest that dietary ganglioside changes ganglioside components made available to peripheral tissues by the gut. Intestinal ganglioside absorption efficiency and ganglioside interactions with basolateral membranes have not been investigated. It is not known whether the intestine takes up gangliosides present in the blood. Understanding this route is essential for intravenous nutrient delivery to patients with compromised bowel function.

1.7.2 Biosynthesis of Gangliosides

Ganglioside biosynthesis takes place along a membrane pathway that starts at the endoplasmic reticulum and finishes at the Golgi apparatus[52,53]. Synthesis of gangliosides is catalyzed by membrane-bound enzymes (glycosyltransferases) and is

assisted by sugar nucleotide transporters[52,53]. Ganglioside synthesis starts with the formation of glycosylceramide at the cytosolic face of the endoplasmic reticulum and its transport to the Golgi apparatus[52,53]. A schematic diagram summarizing the de novo biosynthesis of gangliosides is illustrated (Figure 1-3).

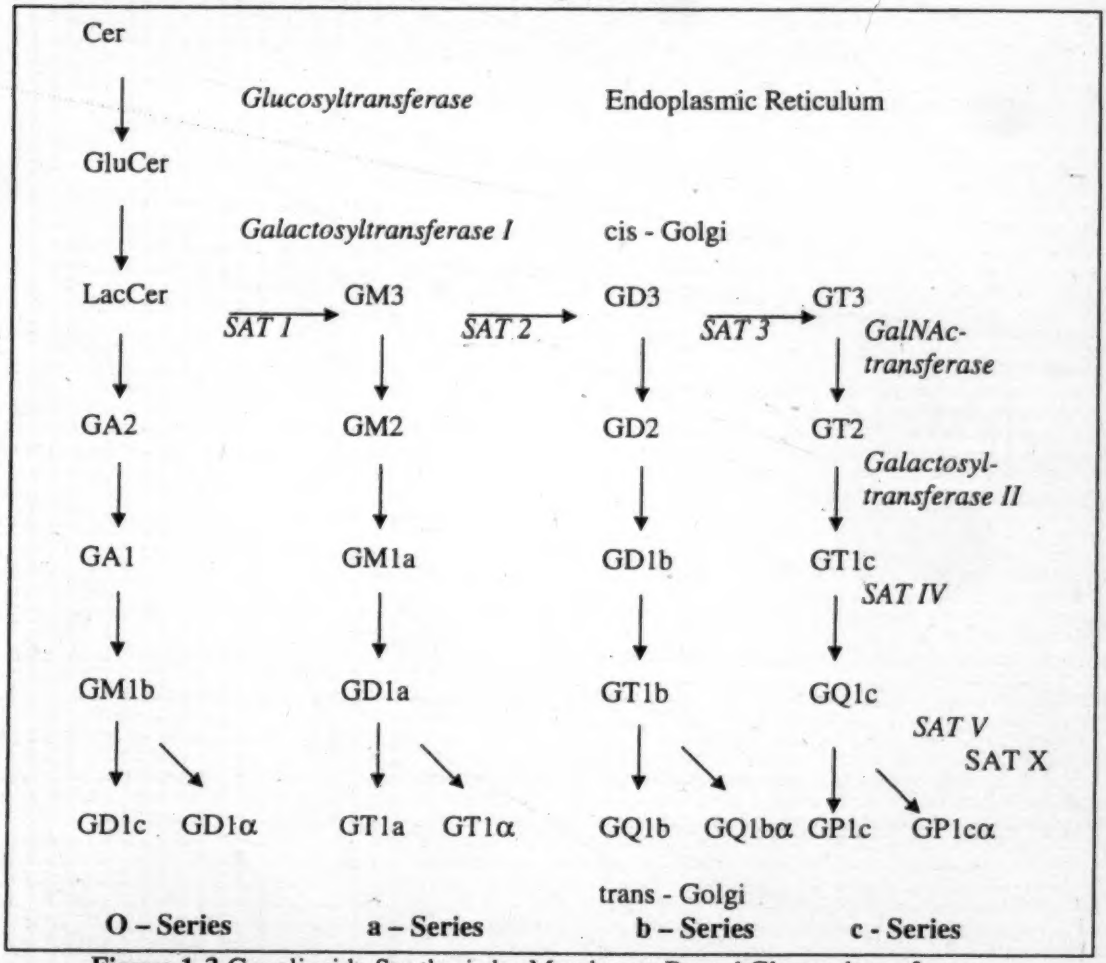


Figure 1-3 Ganglioside Synthesis by Membrane-Bound Glycosyltransferases
SAT = sialyltransferase

The final products of ganglioside biosynthesis leave the trans-Golgi network as budding vesicles that fuse with the plasma membrane[52,53].

1.7.3 Degradation of Gangliosides

Ganglioside degradation occurs on membrane surfaces and consists of sequential removal of individual sugar residues by hydrolytic exoglycohydrolases, assisted by

activator proteins and negatively charged lipids[53,54]. The end product, ceramide, is eventually split into a long chain sphingosine base and a fatty acid by ceramidase[53]. Degradation proceeds along the endosomal-lysosomal pathway[53]. A schematic summary of the degradative pathway for gangliosides is illustrated (Figure 1-4).

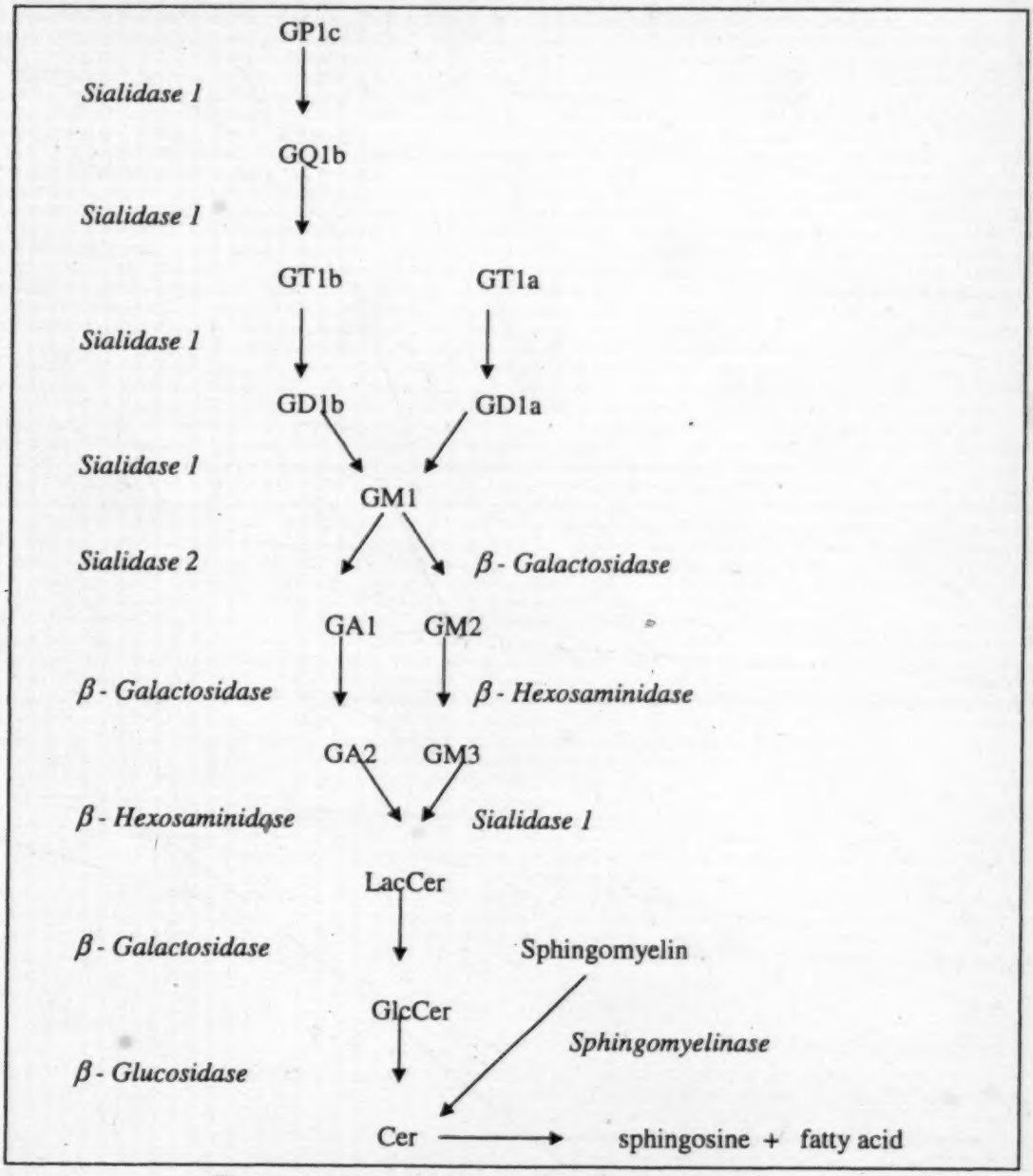


Figure 1-4 Degradative Pathways for Gangliosides

1.7.4 Glycosphingolipid Turnover and Recycling

Ganglioside/glycosphingolipid turnover is determined by the combination of the following events: a) metabolic pathways and fluxes; b) flow of endocytotic and exocytotic routes to which metabolic fluxes are connected; c) rate of plasma membrane turnover reflecting the functional status of the cell; and d) the occurrence of external stimuli that elicit pulses of glycosphingolipid degradation and formation of bioregulatory sphingoid molecules[2]. Both in situ and in vitro experiments have mapped out the metabolic pathway for gangliosides using various radioactive-labeled metabolites to study their metabolic route[53]. Plasma membrane-bound glycoconjugates are susceptible to being glycosylated and deglycosylated by enzymes inserted in the same membrane or approaching the membrane[2]. It is well established at the plasma membrane level, that membrane-bound sialic acid may undergo local turnover[2]. Neural plasma membranes possess a sialosylation/desialosylation system that modulates the degree of ganglioside sialosylation[53]. In developing tissues and some tumors the terminal sialic acid on GD3 is 9-O acetylated[55].

The details of the intracellular destination of endocytosed glycosphingolipids are only partially understood. Evidence shows that the process is mediated by sorting endosomes and presumably by vesicular transport of gangliosides from the sorting endosomal compartment to the Golgi apparatus[53]. For example, it was shown that GM1, radiolabeled on the sphingosine moiety and exogenously added to cells in culture, is transformed into GD1a in the presence of a lysosomal degradation inhibitor[56]. A large body of evidence also proves that endocytosed gangliosides reach the subcellular compartments (late endosomes and lysosomes) where degradation occurs[53]. Inside these organelles the final products of digestion and intermediate bioactive by-products are formed[2]. All or some of these products leave the lysosomes and enter the cytoplasm where they are available for further metabolic processing or biological effects[2]. Direct recycling of ganglioside without metabolic modifications to plasma membranes via transcytotic vesicles or vesicles budded off from sorting endosomes was observed in neuronal cells with GM1[57]. Return of unmodified glycosphingolipids to the plasma membrane with re-synthesis and re-modeling of membrane components is essential to maintain a dynamically constant composition of the cell surface[2]. A schematic of the

subcellular sites of ganglioside absorption, transport, metabolism and trafficking is illustrated (Figure 1-5)[2].

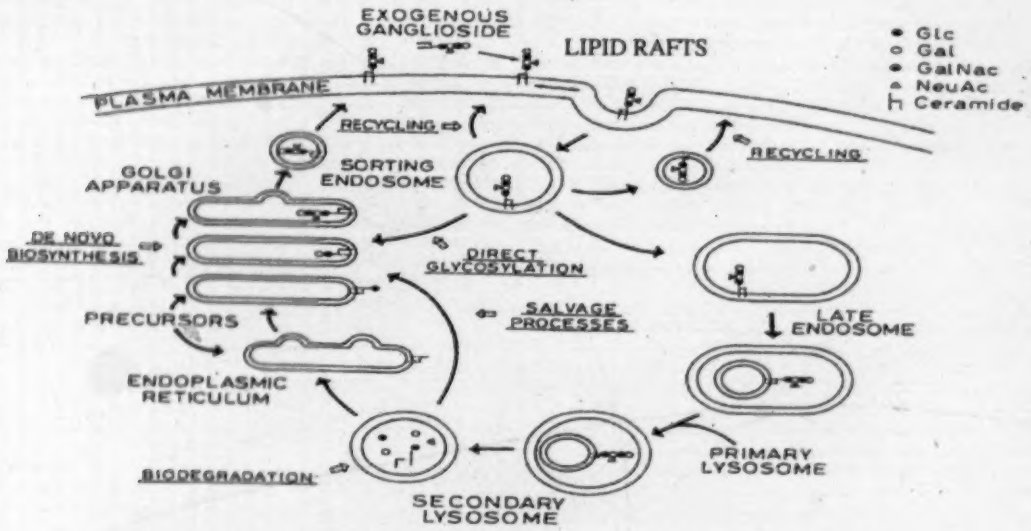


Figure 1-5 Subcellular Sites of Ganglioside Metabolism and Traffic

1.8 Biological Functions of Gangliosides

1.8.1 Cell Recognition, Adhesion and Migration

Gangliosides are known to play an important role in cell recognition, adhesion and migration through carbohydrate-carbohydrate interactions[8]. Gangliosides are amphiphilic molecules with a charged sialic acid group that has a tendency to interact with ions and bind to other molecules[58]. For example, some microorganisms possess lectin or glycoprotein side chains that recognize particular sugar molecules on the ganglioside, which facilitate interactions between microbes and gangliosides[59]. Carbohydrate interactions on the brush border membrane of the developing intestine influence bacteria colonization patterns and susceptibility to pathogen attachment and invasion. For example, pre-term newborn infants fed ganglioside supplemented formula at a concentration of 1.43 mg/100 kcal, were shown to have significantly lower numbers of *E. coli* and higher numbers of *Bifidobacteria* in their feces[4]. Many studies have also shown that gangliosides provide binding sites for a wide range of pathogens including bacteria, viruses, fungi and parasites[60-63]. For example, GM3 acts as a natural receptor in pig intestine for rotavirus[63] and the enterotoxigenic bacteria *Escherichia coli* K99[61].

Moreover, dietary gangliosides have been shown to reduce cyst output and trophozoites in mice infected with *Giardia muris*[64]. Gangliosides also serve as attachment platforms for microbial toxins. For instance, ganglioside GM1 in human intestine[60] and milk[62] provides receptors for *Vibrio cholerae* and heat-labile *E. coli* enterotoxins, thereby acting as a physiological component for protection against these enteric infections.

Carbohydrate interactions involving gangliosides also play a role in cell adhesion and migration during development, tissue repair and cancer metastasis. For example, ganglioside-integrin and ganglioside-selectin interactions have been shown to regulate epithelial cell adhesion to components of the extracellular matrix[65,66]. Research demonstrates that ganglioside interactions with extracellular matrix components inhibit migration and invasion of neuroblastoma cells[67] and promote migration of neurons, glia and corneal epithelial cells in the developing and adult brain and eye, respectively[68,69].

1.8.2 Intestine Barrier Function, Signal Transduction and Pathogen Entry into Microdomains (Lipid Rafts and Caveolae)

The main feature of polarized epithelial cells such as enterocytes is the presence of tight junctions which separate the plasma membrane into an apical and a basolateral domain with specific lipid and protein compositions[70]. These junctions form the permeability barrier between cells and prevent diffusion of nutrients and movement of pathogens through the paracellular route[70]. Tight junction proteins, brush border membrane enzymes/transporters and gangliosides that bind microorganisms and microbial toxins are enriched in glycosphingolipid-enriched areas of the plasma membrane called microdomains[70,71]. Microdomains (caveolae and lipid rafts) are compartmentalized regions of the plasma membrane that are heterogeneously distributed and enriched with clusters of organized lipid and protein molecules that facilitate processes such as cell signaling, cell adhesion and membrane trafficking[8,71]. Caveolae are specialized plasma membrane invaginations and are made up of the protein caveolin[72]. Caveolin proteins stabilize and organize lipid raft components and are necessary for bacteria entry[73]. After internalization, caveolae function in endocytosis, pinocytosis, and regulation of cell signaling processes and transport of cholesterol[72]. Lipid rafts are enriched in cholesterol, glycosphingolipids,

sphingomyelin, phospholipids, GPI-linked proteins and membrane-spanning proteins[74]. Lipid rafts have been implicated in diverse cellular processes including: membrane trafficking, signal transduction, endocytosis, exocytosis, calcium homeostasis, entry of intracellular pathogens and toxins and generation of pathological forms of proteins associated with Alzheimer's and prion disease[74]. Nutrients and microorganisms that associate with lipid rafts avoid lysosomal degradation[70]. Some proteins that are localized within microdomains include membrane receptors for growth factors and signaling enzymes including tyrosine kinase, protein kinase C, phospholipase C, MAP-kinase, adenylyl cyclase, PI3 kinase, calmodulin and nitric oxide synthase[9,58,71,75]. Ca^{2+}-, Na^{+}/ K^{+}- and H^{+}-ATPases are also found in these microdomains associated with membrane transporters[58]. Some signals transduced by gangliosides include cell proliferation, apoptosis and modification of receptor, enzyme and transporter function[58]. Gangliosides often act by modulating protein phosphorylation or interacting with ions. Breakdown products of gangliosides such as ceramide, sphingosine and sphingosine-1-phosphate also regulate cell signaling processes with biological function[9]. For example, ceramide and sphingosine stimulate IL-1-mediated prostaglandin E_2 production[9].

Membrane microdomains play an important role in the attachment and entry of different pathogens and toxins through changes in the spatial organization of tight junctions regulating intestinal barrier function. Microdomain-localized glycosphingolipids including gangliosides are critical for the entry of *E. coli*, *Pseudomonas aeruginosa*, cholera toxin, clostridial neurotoxin, *Helicobacter pylori*, *human immunodeficiency virus* (HIV), Shiga toxin, Rotavirus and *Siman virus 40*[8,76-82]. Cholera toxin and an HIV envelope glycoprotein signal through G proteins in lipid rafts to induce fluid secretion by stimulating chloride secretion and inhibiting Na-dependent glucose absorption[8]. *E. coli*-heat labile toxin binds to GD1b and GM1 in microdomains to transduce signals[82]. These studies suggest that gangliosides have a function in regulating the attachment and entry of certain bacteria, viruses and toxins into cells.

1.8.3 Cell Proliferation, Differentiation and Apoptosis

Gangliosides have been implicated in fundamental cell processes such as growth and differentiation. Growth factor receptors that are regulated by gangliosides include

epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and nerve growth factor (NGF)[71,83]. GM3 and GM1 gangliosides exert an inhibitory effect on tyrosine kinase phosphorylation of EGF, PDGF and FGF receptors[84]. Consequently, they inhibit cell proliferation. Along with NGF, GM1 is responsible for promoting neuritogenesis and differentiation via close contact with a tyrosine kinase receptor to initiate the activation of NGF[85]. The mechanism by which gangliosides influence cell growth and differentiation is an area of investigation. Studies show that gangliosides and ganglioside metabolites, such as ceramide and sphingosine induce apoptosis[9]. Apoptotic stimuli, such as cell death ligands (Fas and TNF), activate sphinomyelinases to generate ceramide and sphingosine at different intracellular locations[9]. Although their contribution to apoptotic cell death is not clearly established, it seems they depend on the apoptotic stimuli used and the cell type studied. In enterocytes, sphingomyelinase has been shown to mediate Fas- and TNF- induced cell death through a dual mechanism involving GD3 generation[86]. An emerging role for ganglioside GD3, as an apoptosis regulator is increasingly recognized due to its ability to recruit mitochondria to cell death pathways[87]. Ceramide is converted into GD3 and GD3 induces apoptosis by disrupting the mitochondrial membrane potential and stimulating cyt c release and caspase activation[87]. Clinically, initiation of apoptosis by GD3 has been used to suppress tumor growth. For example, GD3 has been shown to suppress aberrant crypt formation and adenocarcinoma in mice[10]. Moreover, GD3 enhances the anti-tumor effect of doxorubicin on hepatoma cells[88]. Recently it has been discovered that developing tissues and tumors use a process of 9-O-acetylation of GD3 to protect against apoptotic cell death[89].

1.8.4 Ganglioside Function in Calcium Homeostasis and Signaling

Gangliosides contribute in several ways to the complex calcium regulatory system through modulation of ion channels, transport/exchange proteins and calcium utilizing enzymes[90]. Gangliosides are particularly prominent in neurons, which express unique ganglioside patterns and calcium regulatory mechanisms[90]. Possession of hydrophobic and hydrophilic domains with an associated negative charge on the sialic acid facilitates binding to a variety of amphipathic proteins[90]. Numerous studies

employing exogenous gangliosides have pointed to calcium modulation as the underlying mechanism by which gangliosides assert neuritogenic and neuroprotective effects. For example, treatment of neuronal nuclei with GM1 increases calcium release and stimulates neuritogenesis[91]. In some cases, neuroprotective effects of exogenous gangliosides operate through facilitation of calcium efflux by the plasma membrane Na^+/Ca^{2+} exchanger. For instance, cerebellar neurons pre-treated with GM1 ganglioside reduce the neurotoxic effects of glutamate excitotoxicity by reducing the rise in intracellular calcium[92]. Moreover, treatment of neonatal rats with GM1 ganglioside before a hypoxic insult prevents neurochemical changes (acetylcholine decrease and serotonin increase) and preserves Na^+ K^+ ATPase activity in rat brain and heart ventricular tissue[93,94]. Exogenous gangliosides have also been shown to influence neurotransmitter release through facilitation of depolarization-induced calcium influx into synaptosomes. For example, it has been demonstrated that gangliosides stimulate calcium entry through N-type calcium channels into pre-synaptic terminals and increase synaptosomal release of acetylcholine[95]. Exogenous gangliosides also influence neurotransmitter production by mimicking the effects of calcium/calmodulin on enzymes[96]. For example, the activity of endothelial nitric oxide synthase (eNOS) can be restored by dissociation of the eNOS-caveolin complex by GM1 induced increase in Ca^{2+} concentration[97]. Finally, the importance of negatively charged sialic acid-containing gangliosides in the maintenance of cardiac and smooth muscle calcium levels and muscle contraction is well established[98,99].

1.8.5 Immune System Development and Function

Gangliosides play a role in lymphocyte development and the immune response to inflammatory stimuli. Studies show that GD3 stimulates activation of human peripheral blood T lymphocytes and that GD1a and GD1α are differentiation markers for Th_2 and Th_1 lymphocyte subpopulations, respectively[4]. Gangliosides have also been demonstrated to inhibit antigen presenting cell function[100] and to block the pro-inflammatory NFκβ and iNOS pathways in mitogen-stimulated T lymphocytes[101]. Recently, it was shown that gangliosides can modify the immunological environment during T cell activation by promoting immune deviation in favor of type 2 cell

responses[102]. In the gut mucosal immune system, the role of gangliosides is poorly understood. Feeding weaning mice a semi-purified diet supplemented with a complex mixture of gangliosides promotes early development and significantly higher numbers of Th_1 and Th_2 cytokine-secreting lymphocytes in the lamina propria and Peyer's patches[103]. Recently, our lab demonstrated that dietary ganglioside decreases platelet-activating factor (PAF) content in intestinal lipid rafts and decreases PGE_2, LTB_4, IL-1β and TNF-α production in rats exposed to lipopolysaccharide (LPS)[104]. These studies imply that gangliosides function in gut mucosal immune system development and have anti-inflammatory effects during gut infection.

1.9 Pathological and Pharmacological Function of Gangliosides in Disease

1.9.1 Inherited Sphingolipid Disorders

Glycosphingolipids are degraded in lysosomes by exohydrolase enzymes and sphingolipid activator protein co-factors[54]. The physiological importance of the degradative process in lysosomes is revealed by the existence of at least 40 distinct inherited diseases, the lysosomal storage diseases[105]. The most prevalent subgroup of the lysosomal storage diseases is the sphingolipidoses[106]. These diseases are caused by a deficiency in a single lysosomal enzyme or essential cofactor and result in lysosomal accumulation of one or more glycosphingolipids due to genetic defects in catabolism of sphingolipid containing lipids[106]. Table 1-4 summarizes the major sphingolipidoses[105].

Table 1-4 Summary of Major Sphingolipidoses

Disease	**Enzyme Defect**	**Stored Sphingolipid**
Fabry	α-galactosidase	globotriaosylceramide
Gaucher	β-glucosidase, SAP C	glucosylceramide, GM1, GM2, GM3, GD3
Farber	ceramidase	ceramide, GM3
SL activator deficiency	SL activator protein	glycolipids
GM2 activator deficiency	GM2 activator protein	GM2, other glycolipids
GM2 Gangliosidosis		
Tay-Sachs	β-hexosaminidase A	GM2, other glycolipids
Sandhoff	β-hexosaminidase A + B	GM2, other glycolipids
GM1 Gangliosidosis	β-galactosidase	GM1, GM2, GM3
Krabbe	β-galactosidase	galactosylceramide
Niemann-Pick A + B	sphingomyelinase	sphingomyelin, GM2, GM3
Niemann-Pick C	NPC1, NPC2	cholesterol, GM2, GM3
Metachromic Leukodystrophy	arylsulfatase, SAP B	sulphated glycoproteins glycolipids, GM2
α-Mannosidosis	α-mannosidase	GM2, GM3
Galactosialidosis	protein cathepsin A	GM1, GM2, GM3

SAP = saposin activator protein, SL = sphingolipid

Sphingolipidoses are classified as infantile, juvenile, and adult types according to the onset of disease and level of enzymes[54]. Among those types, the infantile type has the worst prognosis[54]. The clinical presentation of glycosphingolipid diseases is heterogeneous and progressive with neurological symptoms predominating[105]. The pathogenesis of inherited sphingolipid diseases remains poorly understood. Sphingolipidoses that occur naturally in animals and transgenic mouse models will help researchers understand the pathogenesis and develop new treatment strategies[54]. Current treatment strategies for inherited sphingolipid diseases include enzyme replacement therapy, gene therapy, bone marrow transplant and substrate dependent therapy[54].

1.9.2 Neurodegenerative Diseases and Neuron Repair

The natural abundance of gangliosides in developing and adult neurons (10% of total lipid content) indicates a crucial role for gangliosides in the nervous system[107]. Changes in ganglioside composition occur in the mammalian brain not only during development, but also in aging and in several neurodegenerative diseases. The therapeutic potential of gangliosides was first explored more than 30 years ago. Purpura and Suzuki[108] demonstrated that the abnormal accumulation of glycosphingolipids influenced the development and differentiation of neurons. Ceccarelli[109] first demonstrated the ability of exogenous gangliosides to promote the re-growth of damaged neurons. Evidence later confirmed that gangliosides affect multiple neuronal populations (dopaminergic, cholinergic, glutamatergic, serotonergic and noradrenergic neurons)[107] and this prompted studies to examine the potential use of exogenous gangliosides as therapeutic agents for neurological diseases including Parkinson's disease, Alzheimer's disease, ischemic stroke, spinal cord injury and amyotrophic lateral sclerosis (ALS). A small clinical trial of GM1 in patients with Parkinson's disease reported overall improvement in motor function[110]. Studies in animal models have shown that gangliosides facilitate cholinergic reinnervation in Alzheimer's disease and aging[111,112]. On the other hand, GM1 bound amyloid β protein was recently shown to be present in the brain of an Alzheimer's disease patient suggesting that GM1 may facilitate Aβ accumulation in the brain and development of Alzheimer's disease[113]. GM1, GD1b and GT1b have been shown to effectively block glutamate excitotoxicity and apoptosis in vitro and to limit the severity of ischemic brain lesions after experimental stroke[114,115]. The evidence available from acute spinal cord injury clinical trials claims modest benefit with no support for the use of ganglioside treatment to reduce the death rate in spinal cord injury patients[116]. Altered brain ganglioside profiles have been reported in many amyotrophic lateral sclerosis (ALS) patients[117]. It has been hypothesized that gangliosides could promote motor neuron growth and function in ALS patients through generation of neurotrophins[117]. Exogenous gangliosides should continue being used as an experimental tool to promote the recovery of function of various types of neurons following neuronal damage.

1.9.3 Cancer

Gangliosides are involved in various biological events associated with tumor cell growth, migration, adhesion, invasion and formation of metastases[35]. For example, GD3 exerts a dual role in apoptosis regulation by interacting with and recruiting mitochondria to apoptotic pathways while suppressing the activation of cell survival pathways[87]. These novel functions of gangliosides have important consequences in cancer therapy and have prompted the search of strategies to selectively increase GD3 content of tumor cells to maximize cancer therapy[87]. Moreover, gangliosides shed from tumors have been used as diagnostic markers while gangliosides over-expressed on the surface of cancer cells have been selectively targeted with antibodies[35]. Passive immunotherapy using murine or murine/human chimeric monoclonal anti-ganglioside antibodies in their native form or combined with various effectors molecules targeting tumor-specific antigens has been investigated. Most clinical trials involving anti-ganglioside antibodies are in early phase (pilot and phase I) and have been performed in patients with malignant melanoma. Trials performed show acceptable toxicity and partial or complete remission in some patients when using monoclonal antibodies against GD3. The vaccination strategy using native or structurally modified tumor-associated gangliosides in combination with adjuvants is currently the dominant method in clinical trials. Vaccination is a form of active specific immunotherapy, such that the response against the tumor is actively generated by the patient's immune system, and is directed against a particular cellular target or specific membrane antigen[118]. It induces formation of both antibodies and cytotoxic T lymphocytes[35]. In melanoma patients, immunization with anti-idiotypic antibody mimicking GD3 elicited an anti-GD3 response and 60% of the high risk melanoma patients were disease free. Studies involving immunotherapy in combination with other therapeutic modalities are few; however, it has been shown that GD3 sensitizes hepatoblastoma to chemotherapy[119]. One of the challenges that researchers face in killing tumor cells is cancer cell resistance. Recent work demonstrates that tumor cells are able to 9-O acetylate GD3 to protect themselves from apoptosis[89]. Whether gangliosides are able to modulate the activity of multi-drug resistance transporters is still a subject of investigation.

1.9.4 Inflammatory Diseases and Infection

Several early studies established that gangliosides act not only as antigens but also as auto-antigens that play a role in the initiation of self inflammation. In fact, auto-antibodies to gangliosides detected by immuno-staining methods have been described in several autoimmune disorders including Guillain-Barre syndrome, multiple sclerosis, systemic lupus erythematosus, Hashimoto's thyroiditis and insulin-dependent (type 1) diabetes mellitus[120,121]. On the other hand, gangliosides have also been shown to have anti-inflammatory properties in autoimmune diseases by reducing proliferation and activation of immune cells that produce pro-inflammatory mediators. For example, in the classical experimental autoimmune encephalitis model for multiple sclerosis, gangliosides were demonstrated to switch cytokine production from TH_1 to a TH_2/TH_3 phenotype[122]. Moreover, GD3 was shown to down-regulate the pro-inflammatory response of microglia[123]. In addition, changes in ganglioside composition of inflamed tissue may potentially become a marker of inflammation. For example, a recent study identified that excessive amounts of GM3 are present in atherosclerotic lesions and originate from LDL and macrophage/dendritic cell synthesis within the arterial wall[124]. Studies in our lab have demonstrated anti-inflammatory properties of gangliosides during infection. For example, dietary gangliosides fed to weanling rats infected with LPS reduced production of PGE_2, LTB_4, IL-1β and TNF-α[104]. Moreover, dietary gangliosides have been shown to reduce cyst output and trophozoites in mice infected with *Giardia muris*[64].

Necrotizing Enterocolitis

1.10 Definition, Epidemiology and Prognosis

Necrotizing enterocolitis (NEC) is an inflammatory bowel disease of neonates and remains one of the most common gastrointestinal emergencies in newborn infants[125]. Onset of NEC is often within the first three months of life and neonates whom are of extremely low birth weight (< 1000 g) and under 28 weeks gestation are the most susceptible[126]. Full term neonates account for 10% of all NEC cases while premature infants account for 90%[127]. With an incidence rate of 1-5% for all newborns admitted to the NICU[125], a prevalence of 7-14% of very low birth weight infants (VLBW, 500-1500

g)[128] and a mortality rate approaching 20-50%[129], NEC continues to represent a significant clinical problem. In Canada, the incidence rate is 1.8 per 100 live births with a prevalence of 7% of VLBW infants[125]. Advances in obstetric and neonatal care have improved survival rates for smaller, more immature infants, and as more VLBW preterm infants survive the neonatal period, the population at risk for NEC increases[125]. No consistent association between sex, race and rates of NEC has been identified. However, male VLBW infants and black infants are at greater risk of death[130]. Due to inadequate treatments and no effective preventative strategy, an estimated 20-40% of babies with NEC require surgery[125] and 10-30% experience significant morbidity including neurodevelopmental impairment, vision and hearing impairment, failure to thrive, feeding abnormalities, diarrhea, bowel obstruction and short bowel syndrome[125,126,131]. The case fatality rate with surgical intervention is as high as 50%[125]. Necrotizing enterocolitis is also a financial burden to the health care system with yearly hospital charges reported to be as high as $6.5 million in the US[132]. Thus, NEC continues to be an important health issue for preterm neonates.

1.11 Diagnosis

1.11.1 Clinical Signs and Symptoms

The onset of NEC can occur suddenly within a few hours or may be preceded by several days of feeding intolerance[131]. Age at presentation is inversely related to gestational age at birth, with full-term infants often presenting in the first few days of life[125]. Necrotizing enterocolitis affects the gastrointestinal tract and, in severe cases, may have profound systemic impact[133]. Initial symptoms may be subtle and can include feeding intolerance (gastric residuals, bilious vomiting), bloody diarrhea, temperature instability, lethargy, apnea, bradycardia, decreased peripheral perfusion, delayed gastric emptying, ileus, abdominal distension or tenderness and respiratory stress[125,133,134]. Non-specific laboratory abnormalities can include neutropenia, thrombocytopenia, hyponatriemia, hyperglycemia, metabolic acidosis and bacteria or infectious products isolated from blood, urine or stool[133,135]. Serial C-reactive protein could be useful in the management of the disease. C-reactive protein distinguishes stage I NEC from ileus or benign pneumatosis and high levels predict development of complications such as

strictures, abscess or need for surgery[136]. Because early signs of this disease are non-specific, sepsis may be suspected before NEC[125].

1.11.2 Pathological Findings

The ileum and proximal colon are the most commonly affected sites in NEC although any segment of the gastrointestinal tract can be involved including the stomach[137]. Severity of bowel wall necrosis ranges from a small localized mucosal necrosis of a bowel segment to transmural necrosis of the entire small intestine and colon in most severe cases[131]. In more advanced stages of NEC, pathological findings include gastrointestinal bleeding, inflammation, bacterial overgrowth, intestinal distension with multiple dilated loops of small bowel, pneumatosis intestinalis and portal air, intestinal perforation, coagulative necrosis, hypotension, septic shock, pneumoperitoneum and intraabdominal fluid[126,134]. In 1978, Bell and colleagues[138] proposed a system for the uniform clinical staging of infants with NEC. They classified infants as having stage I (suspect), stage II (definite) or stage III (advanced)[138]. Bell's staging criteria for NEC are guidelines for the management of NEC (Table 1-5).

Table 1-5 Bell's Staging Criteria for Necrotizing Enterocolitis

Stage	Systemic Signs	Intestinal Signs	Radiological Signs
Stage IA (Suspected)	Temperature instability, apnea, bradycardia, lethargy	Poor feeding, emesis, ↑ pre-gavage residuals, mild abdominal distension	Normal or intestinal dilation, mild ileus
Stage IB (Suspected)	Same as above	Above and blood from rectum	Same as above
Stage IIA (Proven)	Same as above	Above + absent bowel sounds + mild abdominal tenderness	Intestinal dilation, ileus, pneumatosis intestinalis
Stage IIB (Proven)	Above + metabolic acidosis + thrombocytopenia	Above + definite abdominal tenderness	Above + portal vein gas + possible ascites
Stage IIIA (Advanced)	Above + hypotension, respiratory acidosis, neutropenia	Above + peritonitis, marked distension of abdomen	Above + definite ascites
Stage IIIB (Advanced)	Same as above	Same as above	Above + pneumoperitoneum

Ideally, nutrition intervention begins when an infant has one or more risk factors for developing necrotizing enterocolitis (i.e. preterm birth) or is at an early stage of disease.

1.11.3 Diagnostic Methods

Early diagnosis of gut ischemia and mucosal inflammation/necrosis is crucial in the prevention of NEC or the progression of the illness to late stages requiring surgery and/or bowel resection. An abdominal radiograph and a chest x-ray are used to diagnose gastrointestinal tract abnormalities and changes in the size and shape of the lung and heart, respectively[134,135]. The experimental and clinical methods for early detection of gut ischemia or NEC include serum hexosaminidase, plasma amylin, serum cytosolic β-glucosidase activity, plasma pro- and anti-inflammatory cytokines, serum creatinine kinase isoenzymes, cerebro-splanchnic oxygenation ratio, GI tonometry, rectosigmoid pH monitoring, urinary EGF, D-lactate, or thromboxane, breath hydrogen and MRI[132]. Most of these methods do not have high clinical utility either due to accessibility issues, high costs, and need for expert assistance or due to their poor properties as a diagnostic/ screening test especially in the early stages of NEC. Some infants present so acutely and severely that morbidity or mortality cannot be avoided despite best treatment. Identification of a biological marker for early disease should allow more timely diagnosis and treatment, but no ideal marker has yet been identified. The serum of symptomatic infants tends to contain high concentrations of certain cytokines such as IL-8[139]. Some studies suggest that serum concentrations of fatty acid binding protein in the intestine and liver(I-FABP and L-FABP) could also be used as markers for NEC[140,141]. L-FABP concentrations at the onset of clinical signs are highest in infants later diagnosed with stage I NEC and I-FABP concentrations are highest in infants who later develop stage III NEC[140,141]. More sensitive and accurate imaging studies, such as ultrasonography, could become helpful adjuncts to abdominal films in the diagnosis of NEC[142]. Further research is needed on new approaches for the medical management of NEC that might prevent disease progression and improved surgical outcomes to reduce complications such as short bowel syndrome.

1.12 Pathogenesis

Although the exact etiology and pathogenesis of NEC remains elusive, it is well established that NEC is a complex, multi-factorial disease[126]. Besides pre-maturity, research suggests that other potential predisposing factors are hypoxic-ischemic injury, feeding with formula milk and colonization by pathological bacteria[125] (Figure 1-6).

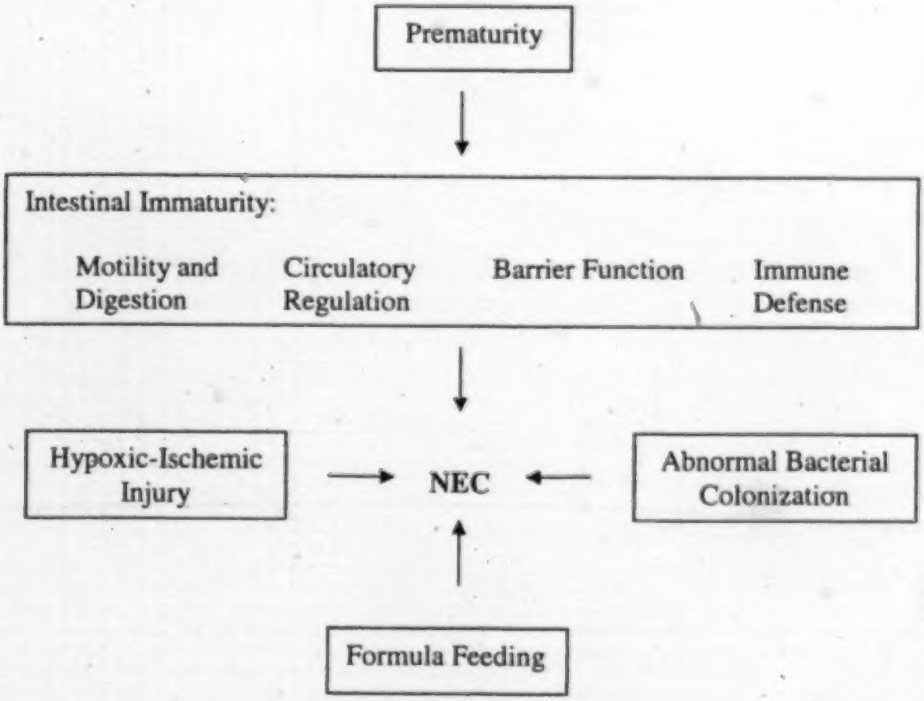


Figure 1-6 Pathophysiology of Necrotizing Enterocolitis (NEC)

Recent studies have shown that carrier state of genetic polymorphisms may be associated with perinatal morbidity, including NEC[143]. The hallmarks of NEC are loss of gastrointestinal motility, disruption of intestinal mucosal integrity and mucosal inflammation, all of which result in the final common pathway, intestinal apoptosis and necrosis[128,144-147]. Several inflammatory and vasoactive mediators including platelet activating factor (PAF), cytokines, nitric oxide (NO), endothelin-1 (ET-1), prostaglandins, leukotrienes and reactive oxygen species are considered to play a synergistic and central role in the final inflammatory pathway leading to NEC[144]. The

consequent breakdown of the mucosal barrier and impaired ability of the mucosa to heal leads to the self-perpetuating vicious cycle resulting in severe NEC, shock, sepsis and sometimes death[132,148,149]

1.12.1 Prematurity

Prematurity is the only factor consistently found in epidemiological studies to be an independent determinant of NEC[126]. Up to 90% of infants with NEC are of low birth weight and the disease is more frequent and severe in those infants with the earliest post-conceptual age[131]. The increased susceptibility is attributed to an immature mucosal barrier and barrier response, changing intestinal microflora and increasing enteral volumes[126,147].

1.12.1.1 Immature intestinal motility, digestion and barrier function

Intestinal motility is a critical factor in clearing antigens presented to the intestinal mucosal barrier from the gut lumen. The time available for absorption depends on the speed of luminal contents. Migratory motor complexes act as "house keepers" to propel luminal components caudally along the length of the small intestine. Immature intestinal motility and digestion may predispose preterm infants to NEC. Fetal studies in both animals and humans suggest that development of gastrointestinal motility begins in the second trimester, but matures in the third trimester[150-152]. Studies of intestinal motility have shown that premature infants can have immature motility patterns when compared with full-term infants and that maternal-fetal disease states that induce fetal hypoxia can further reduce postnatal intestinal motility[153-155]. Immature motility patterns alter normal peristaltic activity and result in overgrowth of anaerobic bacteria in the small intestine with malabsorption of dietary nutrients[147]. In addition, to impaired intestinal motility, premature infants have not yet developed the ability to digest and absorb nutrients and incompletely digested molecules could contribute to intestinal injury[156,157]. Lebenthal and Lee[158] showed that the function of the exocrine pancreas is limited in infants and that pancreatic insufficiency may last through the first year of life. Lack of stimulation of gastric acid and pancreaticobiliary secretions and their resulting proteolysis may adversely affect the intestine by allowing a greater bacterial and/or antigenic load. Thus,

impaired digestion of nutrients, coupled with delayed transit time and bacterial overgrowth could result in intestinal injury with immature host and barrier defenses.

If structural or biochemical components of the intestinal epithelial barrier are not fully developed, bacteria may gain access to deeper tissues and cause inflammation. Intestinal epithelia are joined by tight junctions that regulate intestinal permeability and form by 10 weeks gestation[159]. Studies show that intestinal permeability to macromolecules including immunoglobulins, proteins and carbohydrates is highest in premature infants, particularly in those diagnosed with NEC[144,147]. When fully developed, the intestinal epithelial barrier can allow selective permeability to small ions, absorption of nutrients and control of bi-directional fluid flow. Enterocytes use chloride ions and water secretion to flush unwanted pathogens or toxins from the intestinal lumen. Fetal intestinal secretion and absorption are underdeveloped in preterm infants and mature gradually, under the influence of amniotic fluid, from 26 weeks gestation to full-term[156]. Therefore, pathogens or toxins might not be efficiently washed from the intestinal lumen and could translocate across the leaky intestinal barrier in preterm infants.

Goblet cells are found throughout the small and large intestine. These specialized enterocytes secrete mucins, forming a thick protective layer over the intestinal mucosa. This mucus layer impedes direct microbial-epithelial binding and enhances removal of adherent bacteria[160]. Preterm infants have immature goblet cells. Developmental expression of mucin genes changes throughout the intestine and matches adult pattern expression between 23 and 27 weeks' gestation[161]. Microvilli of immature intestine also have altered glycosylation patterns[162]. Since carbohydrate sequences are recognition and attachment sites for microbes, changes in glycosylation patterns may influence the bacterial colonization pattern of the gut. An immature mucin layer might lead to increased intestinal permeability and enhanced bacterial adherence, potentially breaching the intestinal epithelial barrier and increasing susceptibility to injury.

Another aspect of the intestinal epithelial barrier that may not be functioning correctly in preterm infants is biochemical defenses. Paneth cells, which are specialized secretory enterocytes located at the base of small intestinal crypts, secrete lysozyme, phospholipase A_2 and antimicrobial peptides (also secreted by absorptive enterocytes)

that regulate composition and distribution of different bacterial populations[163,164]. Defensins (α and β) and cathelicidins are the 2 main families of antimicrobial peptides produced by intestinal cells[164]. These anti-microbial peptides have bioactivity against a wide range of microbes including bacteria, viruses and fungi[165]. Some have a pro-inflammatory role and chloride secretory activity[166,167]. A better understanding of how biochemical defense molecules modulate host immune defenses in vivo should contribute to understanding the pathophysiology of NEC.

It is well established that growth factors, growth factor receptors or their related signal transduction pathways are aberrant in the immature intestine. Epidermal growth factor (EGF) is a major trophic factor for the development of the intestine and the EGF receptor has been identified on the basolateral surface of enterocytes[168]. Exogenous infusion of EGF *in utero* has been shown to accelerate the maturation of intestinal enzyme activity as well as stimulate intestinal growth[169]. In the amniotic fluid, there is an increasing concentration of EGF as gestation progresses[170]. In fact, the salivary level of EGF is directly proportional to the gestational age of the infant[170]. Moreover, expression of epidermal growth factor receptor involved in intestinal maturation and restitution is decreased in the preterm infant[131]. Recently, human data suggests a link between EGF production and NEC. Serum and salivary levels of EGF are significantly reduced in infants with surgical NEC[171]. Preliminary studies on the clinical use of EGF report improved epithelial regeneration with no significant toxicities[172].

It is unclear whether the intestinal epithelium of the infant can respond to injury to the same extent as the adult. In animals, infant intestinal epithelium turnover is much slower (4-5 days) than the adult (2 days)[173]. If the same finding holds true in humans, regeneration of injured mucosa in the infant will be much slower than the adult. Trefoil factor peptides (TFF1-3) are part of the protective mechanism operating in the intestinal mucosa and play a fundamental role in epithelial protection, repair and restitution[174]. These secreted peptides have been identified in a site-specific pattern in the gastrointestinal mucosa and their expression has been shown to be up-regulated in early stages of mucosal repair[175,176]. The role of trefoil peptides in neonatal mucosal protection has not been well investigated. Intestinal trefoil factor is developmentally regulated and deficient in the premature neonate[144]. Recent studies demonstrated a lack

of trefoil factor expression in response to NEC in the premature gut[177] and an insufficient proliferative response to reverse the mucosal insult observed in NEC[178]. Thus, impaired restitution of the mucosa may contribute to the cascade of bowel necrosis and generalized sepsis characteristic of NEC.

1.12.1.2 Immature Intestinal Immunity

Although the fetus at term may be sensitized to certain antigens, the fetus does lack a fully functional immune system and has a sterile gastrointestinal tract. Changes occur at, and soon after birth, in order that the immune system of the neonate becomes competent and functional and that the gut becomes colonized with bacteria. Exposure to bacteria during birth and from the mother's skin and the provision of immunological factors in breast milk are amongst the key events that promote maturation of the infant's gut and gut-associated immune system[179]. Dendritic cells play an important role in the initiation of the immune response. Microbial and antigenic-priming of dendritic cells develops different signals that drive the differentiation of naïve Th cells into Th1, Th2 or T regulatory cells[180]. Developmental changes in glycosylation patterns of immature dendritic cells may play an important role in development, maturation and immune regulation[181].

Innate and adaptive immune defense systems are abnormal in developing neonates[144]. A possible mechanism for the pathophysiology of NEC is that reduced inflammatory signaling could allow bacterial overgrowth. Newborns are Th2 polarized and do not respond efficiently to IFN-γ[182]. Moreover, newborn macrophages exposed to LPS are defective in producing pro-inflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-12[182,183]. Interestingly, inhibitory activity to toll receptors in neonatal but not adult plasma has been detected[184]. Neonatal monocyte and T cell production of the anti-inflammatory cytokines IL-10 and TGF-β are developmentally delayed[185]. Preterm infant polymorphonuclear (PMN) counts are lower and premature neonatal macrophages have reduced respiratory burst activation as compared with term newborns[186]. Under conditions of stress, PMN's of term and preterm infants do not function with normal phagocytic and microbicidal activities[187]. PMN's isolated from the blood of term and preterm neonates consistently display diminished chemotactic and

adhesion capacities[188]. It is known that intestinal lymphocytes are decreased in neonates (B and T cells) and do not approach adult levels until 3-4 weeks of life[144]. Newborns also have markedly reduced synthesis of secretory IgA and IgG in response to mitogens, reflecting decreased activity in the intestine[144]. Failure to activate inflammatory pathways in premature infants might prevent induction of anti-apoptotic, cytoprotective factors. Thus, developmental immaturity of the inflammatory response could increase susceptibility to apoptosis when cells are challenged by environmental stress.

Long-term survival requires inflammation as a defense mechanism, however, uncontrolled inflammation results in intestinal barrier damage, translocation of pathogens, further inflammation and tissue damage. Some in vitro studies suggest that immature intestinal cells have a propensity for exaggerated inflammatory responses to pathogenic stimuli and researchers postulate that developmentally deficient expression of the NF-κβ inhibitor Iκβ might allow greater NF-κβ activity[189,190]. NF-κβ is a nuclear transcription factor that enhances the production of inflammatory mediators and is essential for innate immunity, adaptive immunity and cell survival[191]. In the human newborn, PAF-AH activity is decreased and PAF synthesis pathways are increased. This imbalance places the newborn at special risk of an increased PAF response before adequate immune stimuli are developed[131].

1.12.2 Hypoxia-Ischemia

Pathological findings of NEC associated with ischemic events (coagulative necrosis, Table 1-6) and the fact that NEC most commonly occurs in the distal ileum and proximal colon, which make up the watershed area of the superior and inferior mesenteric arteries, suggests that derangement of the circulatory system is involved[131].

Table 1-6 Ischemic Events Associated With Necrotizing Enterocolitis

Perinatal asphyxia
Polycythaemia
Cyanotic congenital heart disease
Patent ductus arteriosis
Medications that ↓ superior mesenteric blood flow (cocaine)
Maternal pre-eclampsia

Preterm neonates are more susceptible to hypoxia and intestinal ischemia because their system for regulating vascular resistance is poorly developed[192]. The most distinguishing feature of the newborn intestinal circulation is its very low vascular resistance due to substantial generation of endothelial derived nitric oxide when compared with endothelin-1[193]. Immature intestine handles the increased metabolic demands of growth by increasing blood flow and oxygen consumption[134]. However, during episodes of cardiovascular stress, infants are less able to raise intestinal blood flow and metabolic demands overwhelm the infant's ability to increase oxygen consumption[134]. Defective pressure flow autoregulation in response to hypotension occurs[192]. Consequently, hypoxia in tissues can occur. Hypoxia increases production of vasoconstrictor endothelin-1 and ischemia/reperfusion compromises production of endothelial derived vasodilator nitric oxide[193]. Thus, an imbalance between endothelin-1 and nitric oxide production by the newborn intestine following an initial ischemic insult might exacerbate existing intestinal ischemia. Whether the hypoxic/ischemic insult is primary, secondary or both an initiating factor and end result remains controversial. One plausible mechanism that is often cited is the "diving reflex", whereby blood flow is preferentially diverted to the heart and brain in preference to less vital organs[125]. Very early descriptions regarding the pathogenesis of NEC suggested a primary or early role for ischemia and hypothesized that a hypoxic/ischemic insult directly damaged the intestinal mucosa disrupting the neonatal gut barrier and promoting bacteria translocation and the inflammatory cascade[145]. Animal models suggest that NEC may not occur without significant reperfusion injury resulting from the generation of oxygen-free radicals at the restoration of blood flow and oxygen delivery after ischemia[133]. Inflammatory mediators may also cause intestinal ischemia by up-regulating endothelin-1 production and the expression of its receptor ET_A[193]. Current studies show a stronger association with prematurity, rapid feeding, abnormal

intestinal colonization and inflammatory mediators than with ischemia[147]. Hypoxia-ischemia might contribute to NEC, but it likely plays more of a secondary role.

1.12.3 Formula Feeding

Enteral feeds have a firm association with NEC as 90-95% of NEC cases occur in infants with initiation/re-initiation of enteral feeds or recent volume advancement[126,144]. Infants receiving hyperosmolar formulas or rapid volume advancements are at greatest risk[144]. Although the mechanism is not well understood, enteral feeding has been reported to contribute to the development of NEC through disruption of mucosal integrity, blood flow and motility and through provision of a bacterial substrate[126,134]. Raising milk intake increases metabolic demands, making it difficult for the infant to expand mesenteric blood flow to meet demands[134]. As a result, intestinal hypoxemia may occur. Increased proliferation of potentially pathogenic bacteria may go on to invade the bowel wall[134]. Although the newborn gastrointestinal tract is sterile at birth, bacterial colonization occurs within hours[134]. Contact with the mother's vaginal flora begins this process, which is further developed by oral feedings and exposure to the environment[134]. In fact, breast fed infants are 10X less likely to develop NEC than formula fed infants, suggesting that breast milk contains multiple bioactive factors that influence host immunity, inflammation and mucosal protection. Breast milk notably increases the diversity of gastrointestinal bacterial colonization and contains immunomodulatory factors such as secretory immunoglobulin A, leukocytes, mucin, lysozyme, cytokines, lactoferrin, growth factors, enzymes, oligosaccharides and polyunsaturated fatty acids not provided in commercially available neonatal formula preparations[144,179]. These factors are capable of inducing mucosal protection and neutralizing potent pro-inflammatory cytokines and phospholipids[179]. Glutamine and nucleotides may help in gastrointestinal cell metabolism[134]. Epidermal growth factor (EGF) can directly improve gastrointestinal function and promote gut maturity[149].

1.12.4 Abnormal bacterial colonization and infection

The well-documented epidemics of NEC and the improvement in incidence and severity following the implementation of strict infection control measures validates the

role of infection in the pathogenesis of NEC[126]. Furthermore, the regions of the intestine that are most often associated with NEC (ileum and proximal colon) have very high bacterial loads. Moreover, no cases of NEC have been described *in utero*, supporting the importance of bacteria colonization in the pathophysiology of NEC[144].

Although several bacterial and viral species have been associated with outbreaks of NEC (*Clostridium* sp, *Klebsiella* sp, *Staphylococcus epidermis*, *Escherichia coli*, *Rotavirus*), no single pathogen has been identified as causative and the ability of the microflora to colonize the epithelium and to ferment unabsorbed nutrients may be more important than the strain itself[137,194]. Recently, early abnormal colonization of stools with *Clostridium perfringens* has been correlated with later development of NEC[195]. Clostridium perfringens has been isolated from 40% of infants with NEC, compared with 13% of controls[195]. Premature infants are especially susceptible to intestinal colonization by pathological bacteria due to their daily exposure to noscomial flora and the likelihood of exposure to antibiotics and steroids on admission to NICU's[196].

Colonization of the gastrointestinal tract of the premature infant differs greatly from that of the healthy term infant[133,144]. Patterns of intestinal colonization also vary according to the type of enteral feeding[127]. The colonization of the hospitalized, premature infant gastrointestinal tract has less species diversity and fewer anaerobic species of *Lactobacillus* and *Bifidobacterium*[133,144]. Breast-fed infants have large amounts of protective, gram-positive *Bifidobacteria* in their intestine, contrasting with formula-fed neonates who are colonized predominantly by potentially pathogenic gram-negative *Enterobacteria*[127]. Gram-positive bacteria yield lactic acid during carbohydrate metabolism, which is readily absorbed from the intestinal lumen, whereas gram negative-bacteria ferment lactose into hydrogen, carbon dioxide and organic acids, producing distension, increased intraluminal pressure, decreased mucosal blood flow and pneumatosis intestinalis[127]. Enteral feeds and poor gastrointestinal motility associated with immaturity may promote stasis and bacterial overgrowth[127]. This microbial imbalance may represent a fertile environment for the pathologic overgrowth, binding and invasiveness of otherwise non-pathogenic intestinal bacterial species capable of triggering the inflammatory cascade with resultant NEC[133]. Recently, inappropriate immunologic responses of premature enterocytes to bacteria colonization

have been implicated in the development of NEC[137]. Reports indicate that pathogenic stimuli including *Salmonella* and *Escherichia coli*, produce exaggerated pro-inflammatory responses in immature intestinal epithelial cells[189,190].

Abnormal expression of pattern recognition receptors that recognize microbial signatures might also affect the way in which the intestine in premature infants responds to bacterial colonization. One of the first pro-inflammatory molecules to cross the intestinal barrier is lipopolysaccharide (LPS), which is a principal component of the outer cell wall of Gram-negative bacteria that recognizes and binds to toll like receptor 4 (TLR4)[145]. Circulating LPS is increased in patients with NEC, which inhibits epithelial restitution and initiates inflammatory cascades within the enterocyte including activation of transcription factor NFκβ and expression of enzymes that produce apoptotic nitric oxide (NO) and pro-inflammatory eicosanoids/cytokines[145] (Figure 1-7).

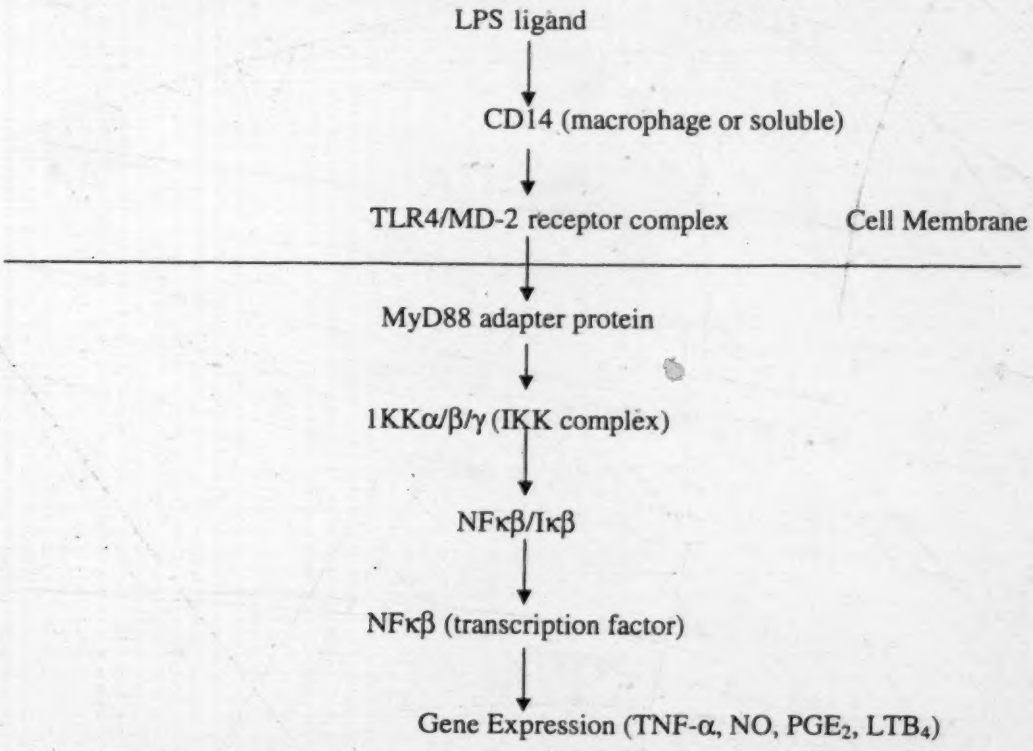


Figure 1-7 LPS-Induced Signaling Pathways Leading to NFκβ Activation

In rats, intestinal epithelial cells up-regulate expression of TLR4 in response to stress-induced production of PAF, suggesting that up-regulation of TLR4 might explain

how NEC develops in this animal model[197]. It remains unclear whether bacterial translocation into submucosa is a prerequisite for disease or whether the activation of the Toll-like receptors from endotoxin and other bacterial cell wall products is adequate to initiate the final common pathway of intestinal injury[144]. For premature infants at risk for NEC, there may be increased passage of bacteria from the gut into the systemic circulation and exaggerated pro-inflammatory responses[134]. Most of the defenses that would normally prevent passage of bacteria across the mucosal barrier – a well-functioning immune system, intact mechanical defenses and normal intestinal microflora are impaired in patients who are at risk for NEC[134]. Gram-negative bacteria translocate to regional lymph nodes and activate resident macrophages to release inflammatory mediators[126]. Bacteria endotoxins can leak into the systemic circulation causing release of inflammatory mediators, intestinal damage, shock and death[126,134].

Commensal bacteria interact symbiotically with the mammalian intestine to regulate the expression of genes important for barrier function, digestion and angiogenesis[198]. Commensal bacteria can inhibit inflammatory pathways and perhaps contribute to the maintenance of homeostasis[199]. In vitro experiments show that a wide range of commensal bacteria can reduce inflammatory signaling in intestinal epithelia by inhibition of the NFκβ signaling pathway[200,201]. Preliminary work suggests that early colonization by probiotics (facultative anaerobes such as *Lactobacilli* and *Bifidobacteria*) reduces the risk of NEC in very low birth weight infants[202,203].

1.12.5 Genetics

Investigation of factors that might cause a genetic predisposition for NEC might eventually allow specific treatments or preventative strategies for the infants most at risk for this disease. Current technology allows for the detection and evaluation of genetic polymorphisms and their influence on disease development. Studies are now emerging which investigate the potential importance of specific polymorphisms for known NEC-associated inflammatory mediators. The presence of genetic variance may contribute to the inter-individual variance of cytokine response to inflammatory stimuli[143]. A family of intracytoplasmic pathogen recognition receptors have been shown to sense invading bacteria and activate gene transcription pathways that regulate

immune and inflammatory responses. In a recent clinical study, VLBW infants with mutations in a member of this family, NOD2, demonstrated increased susceptibility to bacterial sepsis[204]. Genetic polymorphisms of CD14, TLR4 and NOD2 are not associated with NEC in VLBW infants[205]. In preliminary studies, very low birth weight infants with NEC were shown to be less likely to possess the IL-4 receptor α-chain mutant allele compared to infants without NEC[143]. The investigated variant of IL-4 receptor α gene is associated with enhanced transduction of IL-4 signals which shifts the development of lymphocytes to a more pronounced TH_2 state[143]. It is speculated that the elevated number of TH_2 cells in carriers of this genetic polymorphism is a protective factor against the development of NEC[143]. The risk of NEC has also associated with the frequency of the IL-18[607] AA genotype. The frequency of the AA genotype is significantly higher in infants with stage 3 NEC compared to stages 1 and 2[143]. Thus, the presence of the AA genotype may adversely affect the outcome of NEC through altered IL-18 levels, a cytokine that induces IFN-γ and amplifies TH_1 cytokine production and IL-8 accumulation[143]. Another possible genetic factor is the pro-inflammatory cytokine TNF-α. In animal models, pretreatment with anti-TNF-α reduces the incidence and severity of NEC[206,207]. Investigators have not reported a genetic link between TNF-α gene variants and the disease[208].

1.12.6 Vasoactive and Inflammatory Mediators

Bacterial colonization and enteral feeds coupled with damage to and loss of the integrity of the immature gastrointestinal mucosa trigger the final common pathway leading to the development of NEC[133]. Inflammatory mediators are responsible for protecting the body from invading organisms and play a vital role in the pathogenesis of NEC[127]. Inflammation can be initiated by a variety of factors including exposure to the bacterial cell wall product, endotoxin and ischemia reperfusion[144]. The release of potent biologically active phospholipids, cytokines, products of arachidonic acid metabolism, vasoactive mediators, neurotransmitters and reactive oxygen species from the immature and damaged gastrointestinal cells and inflammatory cells amplify the inflammatory response, leading to tissue damage and NEC[133]. Studies of animals and human cell lines

suggest that the balance between pro-inflammatory and anti-inflammatory modulatory factors in premature infants is pro-inflammatory[133].

1.12.6.1 Nitric Oxide

Nitric oxide is a short-lived, labile free radical gas that reacts with a variety of biologically active substances[209]. Such reactions result in both local and systemic effects that modulate the inflammatory response in a variety of tissues[145]. The synthesis of nitric oxide in biological systems is regulated by nitric oxide synthase (NOS), which catalyzes the oxidation of the amino acid L-arginine to release citrulline and nitric oxide[145]. Although diverse molecular reactions of nitric oxide have been identified in physiological and pathological systems, the fastest and most biologically relevant reaction of nitric oxide is with superoxide to produce the potent oxidant peroxynitrite[145]. Peroxynitrite is a key intermediate that is generated at inflammatory sites and is responsible for mediating tissue injury, in part, through lipid peroxidation[145]. Three isoforms of NOS exist: neuronal (nNOS) and endothelial (eNOS), which are calcium/calmodulin dependent and constitutively expressed, releasing physiologically low concentrations of nitric oxide (pM) and the calcium independent inducible isoform (iNOS), which releases toxic concentrations of nitric oxide (nM) in response to infection and inflammatory stimuli[210]. All three isoforms are expressed in the gastrointestinal tract[145,210]. The constitutive forms are expressed by endothelial cells, enteric neurons, gastric epithelial cells and enterocytes[210]. In the gastrointestinal tract, nitric oxide mediates inhibitory nerve-related relaxation of intestinal smooth muscle and plays a role in regulating gut mucosal blood flow, mucosal permeability, intestinal motility and mucosal protection[209,210]. Normal smooth muscle sphincteric function as well as coordinated peristalsis is dependent on the integrity of intrinsic nitric oxide neurons of the myenteric and submucosal networks throughout all regions of the gut wall[209]. Nitric oxide also maintains intestinal microvascular integrity by inhibiting platelet aggregation and leukocyte adhesion[210]. Ontogenic variation in constitutive NOS activity has been observed in different animal species, in humans and in different organs[209]. By contrast, iNOS expression and activity within the intestinal epithelium is normally low, although it may be increased 15-fold after 4 h stimulation with LPS[145].

Nitric oxide and peroxynitrite have anti-microbial properties and play important roles in host defense against pathogens[210]. However, sustained high levels of nitric oxide production promote bacteria translocation following insults such as endotoxemia and ischemia-reperfusion injury[210]. The induction of iNOS mRNA expression by inflammatory mediators has been seen in animal models of NEC and in intestinal resections from patients with NEC where the predominant source of iNOS activity was the enterocytes. Endothelial nitric oxide synthase function is compromised in human intestine resected for NEC[211]. Poorly coordinated production of nitric oxide by nitric oxide synthase isoforms occur during the early phase of the disease and are involved in altered intestinal blood flow, ischemic damage, disassembly of tight junction proteins and impaired healing typically seen in NEC[137]. Research suggests that nitric oxide participates in the pathogenesis of NEC by directly damaging the enterocyte monolayer and by disrupting the ability of the mucosa to repair itself[145]. Extensive apoptosis has been shown in the enterocytes of the apical villi of infants with NEC and this correlates with the degree of nitrotyrosine immuno-staining, a marker of nitric oxide release and tissue reactivity[212]. Toxic concentrations of nitric oxide have also been shown to decrease enterocyte proliferation and inhibit enterocyte migration[145]. It is proposed that peroxynitrite interferes with epidermal growth factor receptor signaling in enterocytes[213].

1.12.6.2 Endothelin-1

Endothelin-1, a potent vasoconstrictor agent, is produced at several sites within the intestine including vascular endothelial cells, submucosal stroma and circularis muscularis layers of the gut wall[193]. Although constitutively produced, endothelin-1 production is increased by a wide range of stimuli including reduced flow rate, hypoxia and inflammatory cytokines[193]. Endothelin-1 generates a profound degree of ischemia that is sustained for hours because of a unique interaction between ET-1 and its receptor[193]. If not balanced by concomitant vasodilatory stimuli, ET-1-induced ischemia can generate hypoxia and tissue death[193]. ET-1 induces vasoconstriction by binding to ET_A receptors present within the newborn intestine and whose activation can generate intestinal tissue damage when excessive amounts of ET-1 are present[193]. Recently,

endothelin-1 was demonstrated to be associated with NEC. It has recently been shown that the tissue concentration of ET-1 is greater in human preterm intestine that demonstrates histologic evidence of NEC[214]. Moreover, it has been demonstrated that arterioles harvested from intestine exhibiting histologic evidence of NEC exhibits vasoconstriction and that the vasoconstriction can be reversed by blocking ET_A receptors[214].

1.12.6.3 Serotonin

Serotonin is an intermediate product of tryptophan metabolism and is primarily synthesized and released by enterochromaffin cells of the intestine (90%) and enteric/brain neurons (10%) in response to calcium influx, physical mucosal stimulation, nutrients, hypoxia and elevations in intraluminal pressure[215]. Levels of serotonin in the gastrointestinal tract are regulated by a serotonin uptake transporter, SERT, present in the mucosa and enteric nerves[216]. The major function of serotonin in the gastrointestinal tract is stimulation of bowel motility, epithelial secretion and vasoconstriction through serotonin receptor binding[215]. Disruption of serotonin homeostasis and signaling is commonly seen in several gastrointestinal motility and inflammatory disorders including bowel obstruction and inflammatory bowel disease[217]. In inflammatory bowel disease, serotonin levels and enterochromaffin cell numbers are increased[217]. The inflamed intestinal tissue releases more serotonin, has a reduced capacity to remove serotonin and the serotonin receptors are desensitized[217]. Some cases of NEC have been associated with maternal use of paroxetine, a long-acting serotonin re-uptake inhibitor[218].

1.12.6.4 Platelet Activating Factor

Platelet activating factor (PAF), an endogenous phospholipid with powerful pro-inflammatory actions, is synthesized by neutrophils, macrophages, endothelial cells and enterocytes in response to endotoxin and hypoxia[134]. Platelet activating factor formation begins with the conversion of a phosphatidylcholine precursor to a biologically inactive intermediate, lysoPAF, under the influence of cytosolic phospholipase A_2 [197]. Subsequent acetylation of lysoPAF at the n-2 position by acetyltransferase completes

PAF synthesis[197]. Platelet activating factor has a very short half life as it is rapidly degraded by PAF-acetylhydrolase[197]. In the human newborn, platelet activating factor synthesis pathways are increased and the activity of the PAF-degrading enzyme PAF-acetylhydrolase is decreased[131]. This imbalance places the newborn at special risk of an elevated PAF response before adequate immune stimuli are developed[131]. Formula does not contain PAF-AH like human milk, leaving susceptible neonates at greater risk for NEC. Platelet activating factor exerts its effects by binding to PAF receptors present on most cells[197]. Interestingly, PAF receptors are most highly concentrated in the ileum, the region of the intestine where NEC is very prominent[148]. Down-stream signaling includes elevation of cytoplasmic free calcium and stimulation of protein kinase C, mitogen-activated protein kinase (MAPK) and NFκβ with production of inflammatory molecules including iNOS, TNF-α, enothelin-1, IL-1, IL-6 and IL-8[197]. PAF also activates pathways that result in caspase activation and apoptosis[197]. Platelet activating factor is one of the most extensively studied mediators of intestinal injury and has been indicated as an important mediator in several animal models and human analyses of NEC[128]. Platelet activating factor infusion causes intestinal necrosis in animals and platelet activating factor receptor antagonists prevent injury following hypoxia, endotoxin challenge and ischemia reperfusion injury[144]. Human patients with NEC show high levels of PAF and decreased levels of plasma PAF-acetylhydrolase with levels correlating with NEC severity[128,148]. In immature or mildly damaged mucosa, the close proximity of bacteria and intestinal epithelial cells aids transcellular permeation of platelet activating factor into the mucosa and local entry of bacteria[134]. Injection of LPS and bacterial invasion leads to increased production of platelet activating factor, release of secondary inflammatory mediators and further mesenteric ischemia and damage causing clinical NEC[134,148].

1.12.6.5 Eicosanoids

Arachidonic acid is a polyunsaturated fatty acid that is liberated from cell membrane phospholipids and serves as a precursor for many immune active lipids, collectively called eicosanoids (oxygenated C20 fatty acids)[219,220]. Classes of eicosanoids that signal in the immune system include prostaglandins, leukotrienes and

lipoxins[219]. The major producers of eicosanoids are platelets, monocytes, macrophages, neutrophils and mast cells, although with the exception of leukotrienes, they are also synthesized by a variety of non-immune cell types[219]. These lipid mediators are not stored in cells rather they are synthesized from arachidonic acid via three major metabolic pathways, either constitutively or in response to cell-specific trauma, stimuli or signaling molecules[220](Figure 1-9).

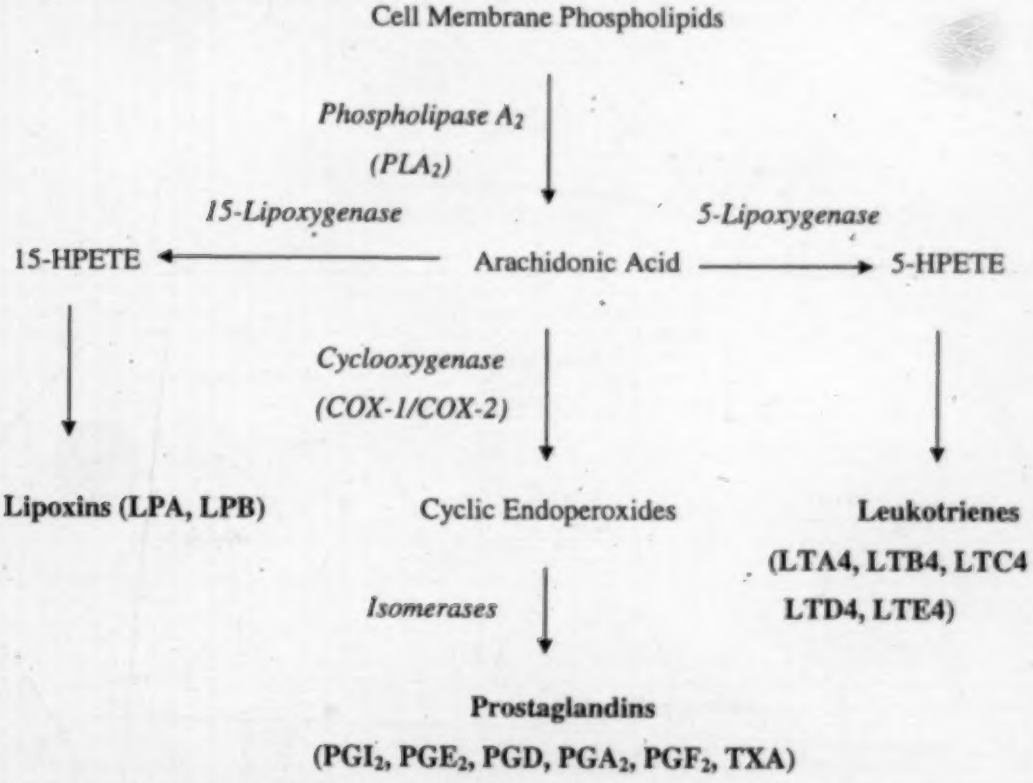


Figure 1-9 Metabolic Pathways of Arachidonic Acid and Eicosanoid Production

The 15-lipoxygenase metabolic pathway results in the production of 15-hydroxyperoxy-eicosatetraenoic acid (15-HPETE) that serves as a precursor for the lipoxins LPA and LPB. Lipoxins exert anti-inflammatory activities through stimulation of macrophage phagocytosis of apoptotic neutrophils and inhibition of natural killer (NK) cell cytotoxicity and pro-inflammatory factor production[221,222].

Prostaglandins are end products of metabolism of arachidonic acid by constitutive and inducible cyclooxygenase isoforms (COX-1 and COX-2, respectively)[220]. The

COX-1 enzyme accounts for basal prostaglandin synthesis for homeostatic regulation while COX-2 is involved in the synthesis of pro-inflammatory prostaglandins[220]. Leukotrienes are generated during the metabolism of arachidonic acid by the 5-lipoxygenase pathway and exert pro-inflammatory effects[219]. Prostaglandins and leukotrienes are emitted from their cell of origin and exert effects in an autocrine or paracrine fashion by signaling through G-protein coupled receptors[219] (Table 1-7).

Table 1-7 Eicosanoid Synthesis and Actions

Eicosanoid	Cell/Tissue Origin	Target Cell/Tissue	Receptor	Action
PGE2	Most cells	Many cells	EP1-EP4	Fever, pain
PGI2	Endothelium	Platelet VSMC	IP	Declumping, vasodilation
PGF2	Uterus	Uterine SMC	FP	Contraction
PGD2	Mast cells	Lung Th2 cells	DP1/DP2	Asthma, chemotaxis
TXA2	Platelets	Platelet VSMC	TPα/TPβ	Aggregation, vasoconstriction
LTB4	Macrophage Monocytes	Neutrophils	BLT1/ BLT2	Promotes chemotaxis
LTC4/LTD4/LTE4	Macrophage Monocytes	Lung SMC	BLT3/ BLT4	Bronchoconstriction
LXA4	Leukocytes	Neutrophil	LXA4 R	Inhibits chemotaxis
LXB4	Leukocytes	NK Cells	?	Inhibits cytotoxicity

1.12.6.6 Cytokines

Pro-inflammatory cytokines are multifunctional proteins produced in response to inflammatory stimuli that communicate to the surrounding tissue the presence of infection or injury. Several pro-inflammatory cytokines that mediate inflammatory cell recruitment through activation and amplification of the immune response in local host defense have been implicated in NEC including tumor necrosis factor-α, interleukin-1 beta, interleukin-6, interleukin-8, interleukin-12 and interleukin-18[223-225]. Anti-inflammatory cytokines modulate the host's inflammatory response and if they fail to achieve their goal, pro-inflammatory mediators can continue, resulting in tissue injury[128]. The anti-inflammatory cytokines IL-4 and IL-10 have been implicated in NEC[128].

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)

The colony stimulating factors are a group of cytokines central to the hematopoiesis of blood cells, the modulation of their functional responses as well as the maintenance of homeostasis and overall immune competence[226]. GM-CSF is produced by a variety of cell types including macrophages, T lymphocytes, fibroblasts, endothelial cells, B lymphocytes, mast cells, eosinophils and neutrophils[227]. In some cases, production of GM-CSF is constitutive as in a number of tumor cells lines; however, in most cases it requires stimulation of the producing cell with other cytokines, antigens or inflammatory agents[227]. All of the biological effects of GM-CSF are mediated via the GM-CSF receptor which signals through MAPK and JAK/STAT pathways[227]. GM-CSF receptor expression is characterized by low number (20-200 per cell) and high affinity[227]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has pleiotropic and widespread effects on hematopoietic cells. It functions to promote the proliferation and maturation of neutrophils, eosinophils, and macrophages from bone marrow progenitors[227]. It also acts as a growth factor for erythroid and megakaryocyte progenitors in synergy with other cytokines[227]. The role of GM-CSF in cell survival results from apoptosis inhibitory mechanisms[227]. In addition to its role in the up-regulation of hematopoietic development, GM-CSF has been shown to have a profound influence on the biological functions of neutrophils, eosinophils, basophils, macrophages, lymphocytes, as well as endothelial cells[227]. These responses are widespread and point to a central role of GM-CSF in inflammation both through the direct activation of effector cells alone or in combination with other cytokines, as well as indirectly, through the stimulation of additional inflammatory mediator production[227]. Some of these biological effects include enhanced antigen presentation, chemotaxis, synthesis of a variety of soluble mediators and enzymes, release of reactive oxygen intermediates and histamines, antibody-dependent cell killing, and phagocytosis which contribute differentially to the immune defenses against bacterial, viral, fungal, and parasitic infections as well as tumor development[227]. Over-expression of GM-CSF leads to severe inflammation[228]. GM-CSF is used clinically to treat neutropenia in cancer patients undergoing chemotherapy, in AIDS patients during therapy and in patients after bone marrow transplant[227,229].

Tumor Necrosis Factor-α (TNF-α)

Tumor necrosis factor-α release is triggered by a number of inflammatory stimuli including endotoxin (LPS), gram positive bacteria enterotoxin, viruses, fungi and parasites[230]. Important cell sources of TNF-α in the gut are macrophages, lymphocytes, NK cells, neutrophils, endothelial cells, smooth muscle cells, intestinal epithelial cells and enteric glia[224,230]. TNF-α exerts its effects by binding to TNF receptors[231]. Binding to the TNF receptor initiates local inflammatory responses through cell activation[231]. TNF-α is released early following injury and leads to a cytokine release cascade of IL-1β, IL-6 and IL-8[224]. It also inhibits release of glucocorticoids and the regulatory cytokines TGF-β and IL-10[230]. Some actions mediated by TNF-α include apoptosis induction, neutrophil activation, neutrophil recruitment, expression of endothelium adhesion molecules, fever and production and release of acute phase proteins, pro-inflammatory cytokines, nitric oxide, PGE2, matrix metalloproteases, PAF and TXA_2[230]. The pro-inflammatory effects of TGF-α are mediated in part through NF-κβ activation[230]. Elevated TNF-α has been detected in full thickness, resected bowel specimens of NEC intestine and in the plasma of babies with NEC[225]. In rat models of NEC, TNF-α induces hypotension, septic shock and severe intestinal necrosis synergistically with LPS[148]. Recently, a monoclonal anti-TNF-α antibody was demonstrated to reduce hepatic and ileal TNF-α production in a neonatal rat model of NEC[206]. Compared with other inflammatory bowel syndromes, TNF-α transcripts are lower in NEC[232]. Furthermore, studies indicate that the majority of TNF-α found in the gut lumen comes from Kupffer cells in the liver[223]. Taken together, these studies suggest that TNF-α plays a less significant role in the inflammatory cascade associated with NEC as compared with other intestinal inflammatory conditions.

Interleukin-1β (IL-1β)

Interleukin-1β release is triggered by a variety of stimuli including microbial products, inflammation and TNF-α[224,233]. Important cell sources of IL-1β in the gut are macrophages, neutrophils, intestinal epithelial cells, endothelial cells, fibroblasts, dendritic cells, smooth muscle cells and enteric glia[224,233]. Interleukin-1β exerts its

effects by binding to the IL-1 receptor and activating the transcription factor NF-κβ[233]. Some actions mediated by IL-1β include macrophage activation, neutrophil recruitment, expression of endothelium adhesion molecules, fever and production and release of acute phase proteins, IL-6, IL-8 and PGE_2[224,233]. Elevated IL-1β has been detected in full thickness specimens of NEC intestine[225]. Studies measuring plasma/serum IL-1β in NEC babies have not consistently reported elevated levels[139]. The difference in results may suggest that IL-1β is more predominant in the intestinal tissue in patients with NEC.

Interleukin-6 (IL-6)

Interleukin-6 release is triggered by a variety of stimuli including microbes, microbial products, TNF-α and IL-1β[224,234]. Important cell sources of IL-6 in the gut are macrophages, endothelial cells and intestinal epithelial cells[234]. Interleukin-6 exerts its effects by binding to the IL-6 receptor that signals through the STAT-4 pathway[234]. The IL-6 receptor is only expressed on hepatocytes and some leukocytes[234]. Some actions mediated by IL-6 include production of acute phase proteins, B cell growth, antibody production, T cell proliferation and enhanced activity of hematopoietic growth factors such as granulocyte-monocyte colony stimulating factor (GM-CSF)[224,234]. Anti-inflammatory effects of IL-6 include production of tissue inhibitors of metalloproteinases and inhibition of superoxide production[235]. High levels of umbilical cord IL-6 have been associated with neonatal disease processes including NEC and systemic inflammatory response syndrome[236]. Elevated IL-6 has been reported in the plasma and stool of babies with NEC[128]. A study that looked at IL-6 mRNA expression in surgical intestine specimens from babies with NEC did not find a difference in comparison to control specimens[225]. Since IL-6 plays a dual role in inflammation it may serve as an anti-inflammatory mediator despite being correlated with increased morbidity and mortality in NEC patients.

Interleukin-8 (IL-8)

Interleukin-8 synthesis and release is triggered in response to various stimuli including LPS, TNF-α and IL-1β[237]. Important cell sources of IL-8 in the gut are

macrophages, endothelial cells, intestinal epithelial cells and fibroblasts[238]. Interleukin-8 exerts its effects by binding to chemokine receptors CXCR1 and CXCR2 that signal through phospholipase c and PI3-kinase, respectively[237]. Some actions mediated by IL-8 are attraction of neutrophils and basophils to the site of inflammation, neutrophil activation and migration into tissues and production of acute phase proteins[238]. In intestinal specimens from patients with NEC, IL-8 mRNA expression was up-regulated, correlated with disease severity and was limited to areas with histological inflammation[128,239]. Similarly, plasma IL-8 levels are elevated in infants with NEC and levels correlate with clinical severity[128]. The vulnerability of the premature infant to develop NEC may, in part, be explained by the excessive inflammatory response shown by fetal enterocytes compared to more mature enterocytes[190]. When exposed to inflammatory stimuli, fetal intestinal cells exhibit a greater IL-8 response compared to mature intestinal cells[190]. This exaggerated response may partly be explained by the developmental down regulation of Iκβ, an inhibitor of NF-κβ[189].

Interleukin-12 (IL-12)

Interleukin-12 synthesis and release is the early response to bacteria, bacterial products and viruses[240]. Important cell sources of IL-12 in the gut are macrophages, neutrophils, B cells and dendritic cells[240]. IL-12 exerts its effects by binding to IL-12 receptors present on T cells and NK cells[240]. Some actions mediated by IL-12 include IFN-γ production, TH1 and NK cell proliferation, cytotoxic T lymphocyte and TH1 cell differentiation, macrophage activation and production of complement-fixing antibodies[240]. Several studies have identified putative NF-κB sites in the promoter regions of the IL-12 p40 genes[241]. IL-12 is a potentially important cytokine in the development of NEC. Halpern[223] localized IL-12 via immunohistochemistry to monocytes in the intestinal mucosa and lamina propria and correlated IL-12 positive cells with tissue damage in a neonatal rat model of NEC.

Interleukin-18 (IL-18)

Interleukin-18 is a cytokine that shares structural and functional properties with IL-1 and is pro-inflammatory inducing production of TNF-α and IL-1β[242]. Interleukin-18

synthesis is triggered by LPS, Fas ligand and gram positive bacteria exotoxins[243]. Important cell sources of IL-18 in the gut are macrophages, dendritic cells and intestinal epithelial cells[243]. IL-18 exerts its effect by binding to the IL-18 receptor present on macrophages, neutrophils, NK cells, endothelial cells, smooth muscle cells and lymphocytes[243]. IL-12 upregulates the IL-18 receptor on lymphocytes[243]. Binding to the IL-18 receptor results in NF-κβ activation. Some actions mediated by IL-18 include IFN-γ production, enhanced NK cell cytotoxic activity, B cell antibody production, macrophage production of IL-8, activation and migration of neutrophils, phagocytosis and integrin expression[243]. IL-18 can promote TH1 or TH2 lineage maturation depending on the underlying genetic influence and cytokine environment. The risk of NEC has been associated with the frequency of the IL-18^{607} AA genotype[143]. Recent data imply that interleukin-18, in the absence of interleukin-12, may facilitate the development of Th2 responses[242]. IL-18 is also essential to host defense against a variety of infections[243] and is potentially important in the development of NEC. Immunohistochemistry reveals the presence of IL-18 in intestinal epithelial and lamina propria cells which correlates with the degree of tissue damage in a neonatal rat NEC model[223]. Depending on the surrounding environment, IL-18 may play a destructive or protective role in NEC.

Interleukin-4 (IL-4)

Interleukin-4 is pleiotropic, immunoregulatory cytokine produced by Th2 cells, mast cells, B cells and stroma cells[224,244]. IL-4 displays a wide variety of effects on B cell growth and differentiation, T cell growth and regulation, hematopoietic cells and differentiation of CD4+ T cells into TH2 cells and is a key regulator in humoral and adaptive immunity[224,244]. IL-4 induces B cell class switching to IgE and upregulates MHC class II production[224,244]. IL-4 is known to promote Th2 type responses and to exert immunosuppressive effects on macrophages including the suppression of pro-inflammatory cytokine production[224,244]. Although data are not available about the importance of IL-4 in NEC, isolated lamina propria mononuclear cells from the inflamed intestinal mucosa of children with chronic inflammatory bowel disease express and secrete IL-4 in lower amounts than control cells[245]. In preliminary studies,

very low birth weight infants with NEC were shown to be less likely to possess the IL-4 receptor α-chain mutant allele compared to infants without NEC[143]. The investigated variant of IL-4 receptor α gene is associated with enhanced transduction of IL-4 signals which shifts the development of lymphocytes to a more pronounced Th2 state[143]. It is speculated that the elevated number of Th2 cells in carriers of this genetic polymorphism is a protective factor against the development of NEC[143].

Interleukin-10 (IL-10)

IL-10 is the most important regulatory cytokine in the intestine and is primarily synthesized by Th2 cells, monocytes and B cells[244]. Mononuclear production of anti-inflammatory mediators such as IL-10 is diminished in the newborn when compared to the adult, with preterm infants synthesizing less than term infants[246,247]. It is postulated that this phenomenon allows for persistent up-regulation of the inflammatory response and therefore increased susceptibility in the preterm neonate to long-term tissue damage after acute inflammatory conditions[248]. Interleukin-10 has been implicated as an inhibitor of pro-inflammatory cytokine production and of several accessory cell functions of the macrophage, T cell and natural killer (NK) cell lines[244]. Kuhn[249] demonstrated that IL-10 deficient knockout mice were predisposed to developing inflammatory colitis, suggesting that IL-10 works to counterbalance the response to enteric inflammatory stimuli. In fact, intraperitoneal IL-10 injections in a mouse model of ischemia/reperfusion injury reduced local and systemic inflammatory reactions[250]. Edelson[139] noticed significantly increased concentrations of IL-10 with severe NEC. IL-10 has also been shown to decrease the production of metalloproteinases[251] and suppress iNOS mRNA and nitric oxide expression in small bowel, liver and serum[252]. These findings indicate that IL-10 is a strong counter regulatory cytokine and that the potential of IL-10 to provide therapy in the setting of NEC is high. Perhaps high levels of IL-10 in severe NEC are the body's response to dampen the inflammatory response.

1.12.6.7 Reactive Oxygen Species

One of the major endogenous sources of reactive oxygen species (ROS) in the intestine is the xanthine dehydrogenase/xanthine oxidase (XD/XO) system[253]. Xanthine

dehydrogenase (XD), the precursor of XO, is constitutively and abundantly expressed in the intestinal villus epithelium, which catalyzes the conversion of hypoxanthine to xanthine, coupled with the reduction of NAD^+ to NADPH[254]. Because XO uses molecular oxygen rather than NAD^+ as an electron acceptor and thereby generates superoxide, XD to XO conversion (during ischemia) has been suggested to play the central role in intestinal reperfusion injury[253]. Following PAF challenge, it is the ileum that shows the most dramatic XD to XO conversion[254]. The central role of xanthine oxidase and ROS in causing the injury is supported by pre-treatment with allopurinol, a xanthine oxidase inhibitor, which largely prevents PAF-induced bowel necrosis[255]. Infusion of superoxide dismutase plus catalase also alleviates the injury[255]. In a piglet model of NEC, the level of the tissue antioxidant, α-tocopherol (vitamin E) was low in formula compared to colostrum fed piglets[137]. Thus, infants with NEC are under oxidative stress and may benefit from exogenous sources of antioxidants to replenish limited supplies.

1.13 Necrotizing Enterocolitis Models

There are a number of accepted models used to study NEC and the cytokine cascade. These models serve to create necrotic bowel in animals to simulate that in the newborn child. Lipopolysaccharide (LPS), platelet activating factor (PAF) and tumor necrosis factor-α are often used to create intestinal ischemia. LPS is thought to mimic the bacterial overgrowth in the intestinal lumen and PAF and TNF-α cause a hypotensive response and shock[148].

Many animal models can simulate NEC, but often do not contain the aspect of prematurity that is seen in human NEC. The most physiological animal model that most closely resembles human NEC entails removing rat pups from the maternal uterus, exposing them to maternal milk, and stressing them with asphyxia, gram negative bacteria colonization and artificial formula feedings[256]. After a few days of life, the rat pups begin to exhibit signs of NEC including intestinal distension and bloody diarrhea.

Other models have been described that do not physiologically resemble human NEC, but aid in the study of the disease process. These include inducing hypoxia for 5 min followed by 10 min with 100% oxygen[257], hypoxia for 50s followed by cold

exposure[258], superior mysenteric artery clamping with or without PAF[259], intraarterial injection of TNF-α[260], and placing rats into a 100% nitrogen or 10% oxygen environment[148]. Finally, a rat model has been described by Chan[261] who created ischemia by increasing intraluminal pressure and injecting *E. coli* into the lumen.

In addition to *in vivo* animal models, various *in vitro* models have been created. The cell lines are often intestinal-derived and immortal such as CaCo-2, a human colon carcinoma cell line[262]. Inflammatory stimulants such as LPS and pro-inflammatory cytokines can be added to cell cultures which can then be analyzed to determine the presence or absence of specific cytokines. In addition, cells can be studied with regards to permeability, viability and expression of inflammatory markers after addition of certain stimulants or creation of hypoxic environments. Paracellular conductance can be assessed by measuring both trans-epithelial resistance (TER) and determining the rate of permeation of radiolabelled, hydrophilic probes between mucosa and serosa compartments of vertical diffusion chambers. It is unfortunate that primary cultures of human enterocytes have a limited life span (hours) in culture and therefore have not been useful as a model.

1.14 Symptomatic Treatment and Surgery

Due to the limited understanding of the fundamental biological processes that underlie the development of NEC, there is no cure for this devastating pediatric disease[145]. Symptomatic treatment of the infant with NEC begins with prompt recognition of the diagnosis and medical stabilization[126,133]. The treatment of NEC is based on the severity of the disease and is directed toward reduction of factors that aggravate the disease, treatment of infection and support of respiratory and cardiovascular systems[263]. Blood pressure should be closely monitored, all enteral feedings and medications should be discontinued and decompression of the gastrointestinal tract with placement of a gastric tube should proceed to evacuate residual air and fluid[133]. Rapid volume expansion with isotonic fluids may be necessary to reverse hypotension as well as frequent monitoring of blood glucose levels[133]. An intravenous infusion of total parenteral nutrition should begin during the 10-14 day bowel rest period[133]. The reinstitution of feedings generally is done in a slow and

cautious manner, using an elemental formulation to allow for optimal absorption of all nutrients and to avoid further potential injury to the intestinal mucosa[131]. Broad-spectrum antibiotics including ampicillin and an aminoglycoside should be started as soon as cultures have been obtained[263]. With the increasing prevalence of infections from coagulase-negative staphylococcus, vancomycin may be used instead of ampicillin[126]. Anti-microbial choices should be guided by local resistance patterns[126,263]. Adjunctive therapy includes cardiovascular support (volume expansion with packed red blood cells), pulmonary support (oxygen and ventilation) and hematological support (blood product transfusion) as clinically indicated[125,263]. Indications for surgical intervention include peritoneal free air and signs of intestinal perforation[133]. Surgical intervention frequently results in resection of areas of necrotic bowel and exteriorization of viable ends (multiple ostomies) to allow for continued bowel decompression[126,133]. Recently, primary peritoneal drainage has been proposed as an alternative to surgical treatment. NEC STEPS and NET, prospective multi-centre randomized controlled trials, are currently underway to examine the effectiveness of primary peritoneal drainage versus laparotomy as primary therapy for perforated NEC in VLBW infants[264,265].

1.15 Prevention

Strategies to prevent NEC fall into two major categories: those with probable or proven efficacy and those that are experimental with unproven efficacy[126] (Table 1-8).

Table 1-8 Strategies to Prevent Necrotizing Enterocolitis

Evidence-Based Support for Efficacy	Limited Data to Support Efficacy
Breast feeding	Cautious advancement of feedings
Trophic feeding	Fluid restriction
Antenatal steroids	Oral immunoglobulins
Enteral administration of antibiotics	L-Arginine supplementation
	Polyunsaturated fatty acids
	Acidification of milk feeds
	Probiotics, prebiotics and postbiotics
	Growth factors and erythropoietin
	Free radical scavengers

The most effective preventative strategies should improve both short-term and long-term outcomes for VLBW preterm infants and address the problems of prematurity.

1.15.1 Human Milk

Human milk has been reported to reduce the incidence of NEC by up to 10 fold compared with infant formula whether using mother's own or donor milk[266]. Breast milk also reduces the severity of NEC[132]. The protective effect of breast milk has been correlated with its anti-inflammatory components (IL-10), growth factors (EGF), erythropoietin, lysozyme, immunoglobulins as well as pre- and probiotics that modulate intestinal microflora composition to the advantage of the host[179,267,268]. Research looking at a gut-stimulation, or gut-priming protocol has demonstrated potential benefits of promoting maturation of the gut by introducing early feedings with human milk[127]. The activity of acetyl hydrolase (PAF-AH), an enzyme that degrades PAF, is lower in neonates under 3 weeks of age than at any other time[269,270]. The additional presence of PAF-AH activity may also partly explain the protective effect of breast milk, as infant formulas do not contain it[132]. Whether preterm human milk reduces the incidence of NEC is not clear at present[132]. Despite its advantages, it is important to appreciate that human milk alone will not eliminate NEC as cases are reported in neonates who have been breast-fed exclusively with human breast milk[132].

1.15.2 Trophic Feeds

Initiation of trophic feeds, small volumes of breast milk or formula, may overcome gut atrophy and inflammatory responses associated with prolonged bowel rest. Trophic feeds improve the activity of digestive enzymes, enhance the release of digestive hormones and increase intestinal blood flow and digestive motility in premature infants[271]. In addition, infants given early trophic feeds seem to have better feeding tolerance, improved growth, reduced period of hospitalization and decreased likelihood of sepsis compared with infants who are not[271]. Furthermore, early trophic feeds do not increase susceptibility to developing NEC. However, studies have not yet clearly delineated the best feeding strategies for premature infants[271].

1.15.3 Antenatal Glucocorticoids

Antenatal glucocorticoid therapy has beneficial effects by suppressing inflammation and promoting gastrointestinal maturation and function including reduced mucosal uptake of macromolecules, decreased colonization with aerobic bacteria, reduced bacterial translocation and increased activity of enzymes such as lactase, maltase, sucrase and Na/K-ATPase[272,273]. A significant reduction in the incidence and risk of NEC following antenatal glucocorticoid therapy has been reported in several large, randomized control trials[274,275]. Mortality rate was also lower and there were fewer indications for surgical intervention[276]. Antenatal glucocorticoids have been reported to alter immune system development in very premature infants[277]. Mothers with the presence of infection or a condition that may compromise blood flow to the fetus (ex. pre-eclampsia) during pregnancy may be at risk of delivering a premature baby and may potentially benefit from early use of glucocorticoids. Thus, antenatal glucocorticoid therapy is a simple and effective strategy for global prevention of NEC and more research should be done to investigate potential impact on development.

1.15.4 Enteral Antibiotics

Enteral antibiotics have been used as prophylaxis against NEC in low birth weight and preterm infants given the role of bacterial colonization in the pathogenesis of the illness. A systemic review and meta-analysis has reported that the administration of prophylactic enteral antibiotics resulted in significant reduction in NEC[278]. The trend towards a reduction in deaths was not significant[278]. The possible harmful effects of prophylactic antibiotics including the development of bacterial resistance and alteration of the natural microflora make it difficult to recommend this strategy for prevention of NEC.

1.15.5 Standardized Feeding Regimens (Cautious Advancement of Feedings)

Inter-centre differences in clinical practice involving feeding regimens are significant factors linked to the prevalence of NEC in VLBW neonates[279]. A relationship between the rate of feeding advancement and an increased incidence of NEC exists[280]. A significant decline of 87% in the incidence of NEC and 29% in the

risk of developing NEC was reported following implementation of a standardized feeding regimen in the form of clinical practice guidelines[281,282]. Parenteral nutrition coupled with minimum enteral feeding is the approach commonly advocated for the initial nutritional management of high risk infants and helps protect against NEC[283].

1.15.6 Fluid Restriction

Excess fluid has been implicated in the pathogenesis of NEC. A systemic review and meta-analysis indicates that restricted water intake significantly increases postnatal weight loss and significantly reduces the risk of NEC[284]. Careful restriction of water intake (meeting the physiological needs without allowing significant dehydration) could be expected to decrease the risk of death from NEC without significantly increasing the risk of adverse consequences.

1.15.7 Probiotics

Since bacterial colonization can affect the course of many intestinal diseases, probiotics are emerging as a promising therapy. Probiotics are living microorganisms, which upon administration in sufficient numbers colonize the gut and exert health benefits beyond basic nutrition on the host[285]. As components of infant formula, typically used probiotic microorganisms are members of the genera *Lactobacillus*, *Bifidobacterium*, *Saccharomyces* and to a lesser extent *Streptococcus*. The beneficial effects of probiotics range from changes in intestinal permeability and enhanced mucosal IgA responses to an increased production of anti-inflammatory cytokines and protection of the mucosa against colonization from pathogens[286]. *Bifidobacteria* are the most common organisms recovered from the gastrointestinal tract of breast-fed neonates. Given the role of inappropriate gastrointestinal colonization by bacteria in the pathogenesis of NEC, probiotics may be beneficial in the prevention of NEC. Several studies have used different strains of probiotics and different administration regimens (length of treatment and dose) in preterm infants. None of the trials have reported adverse effects and no episodes of pathogenic infection caused by a probiotic organism have been observed[202,203,287,288]. Clinical trials show that probiotic supplements (*Lactobacillus acidophilus*, *Bifidobacterium infantis*, *Bifidobacterium bifidus* and

Streptococcus thermophilus) reduce the incidence and severity of NEC[202,203,289]. Larger clinical trials are necessary to confirm the safety and efficacy of this promising intervention to better define the benefits and risks for premature infants before wider use can be recommended.

1.15.8 Prebiotics

Another potential preventative strategy is to administer prebiotics, non-digestible dietary supplements, such as long chain carbohydrates or mucins, which promote proliferation of beneficial commensal bacteria[290]. Preliminary studies show increased *Bifidobacterium* stool colonization and decreased pathogenic bacterial colonization in preterm infants fed with formula containing prebiotics (90% short chain galacto-oligosaccharide, 10% long chain fructo-oligosaccharide) compared with infants fed control formula[291]. Furthermore, prebiotic treatment may have a positive effect on host immune function[292]. Because prebiotic supplements do not contain live microorganisms, they carry less risk of infection than probiotic therapies. However, prebiotic administration has been associated with unwanted (but reversible) side effects such as flatulence, bloating and diarrhea[290].

1.15.9 Postbiotics

Another potential therapy involves bacterial metabolites or postbiotics, such as butyric acid, a short-chain fatty acid produced by commensal bacteria in the colon through anaerobic catabolism of complex carbohydrates. Butyrate is a major energy source for colonic enterocytes and has a widely recognized but poorly understood role in intestinal growth and differentiation[293,294], inflammatory suppression[295] and apoptosis[296]. Butyrate and other small molecule products might generate some of the beneficial effects of the normal flora (and exogenous probiotics and prebiotics), and could be a safe alternative therapeutic strategy. Butyrate has been administered with limited success in human inflammatory bowel disease[297], but there are as yet no studies in neonates.

Other products of commensal bacteria can induce protective responses that promote intestinal health. The beneficial effects of probiotic bacteria can be replicated by

treatment with isolated microbe-associated molecular patterns (MAMP's)[298]. A MAMP is a molecular sequence or structure in any pathogen-derived molecule that is perceived via direct interaction with a host defense receptor[299]. For example, in mice unmethylated probiotic DNA ameliorates colitis[298]. Oral administration of inactivated probiotics (heat-killed commensals) or bioavailable toll-like receptor ligands could potentially induce beneficial TLR-mediated protective effects without carrying the infectious risk of probiotic therapies.

1.15.10 Arginine Supplementation

Endothelial nitric oxide is an anti-inflammatory agent and vasodilator that is involved in the maintenance of intestinal vascular permeability, mucosal integrity and barrier function[145,210]. The plasma levels of the amino acid arginine, a substrate for nitric oxide synthase, have been shown to be low in neonates with NEC[300,301]. Arginine supplementation has recently been shown to reduce the incidence of all stages of NEC in a randomized, double blind, placebo controlled trial in preterm neonates[302]. Whether the beneficial effects of arginine supplementation in prevention of NEC are related to synthesis of glutamine or to its free radical scavenging action is currently unknown[303,304]. Guidelines have not been established for the safety and efficacy of L-arginine at doses above standard dietary practices in NEC[305].

1.15.11 Free Radical Scavengers (Anti-Oxidants)

Free radicals have been implicated in several neonatal disease processes including NEC[306]. A human recombinant superoxide dismutase is currently available and has been shown to prevent damage and attenuate eicosanoid release in a rabbit model of NEC[307,308]. The anti-oxidant vitamin E has been shown to reduce lipid peroxidation and intestinal lesions in a neonatal rat model of NEC induced by hypoxia-ischemia[307,308]. More studies on the therapeutic role of anti-oxidants in NEC should be done.

1.15.12 Acidification of Gastric Contents

Preterm neonates are often hypochlorhydric and enteric, Gram negative bacteria often colonize their stomachs, especially after gavage feeding[309]. Carrion [310] have

documented that acidifying the feedings of preterm neonates to a pH low enough to inhibit gastric bacterial proliferation significantly lowers the risk and incidence of NEC.

1.15.13 Polyunsaturated Fatty Acids

Phosphotidylcholine (PC) is a major phospholipid constituent of mucosal membranes and the fatty acid component of PC, arachidonic acid, is a substrate for intestinal vasodilatory and cytoprotective eicosanoids[132]. Long chain polyunsaturated fatty acids (PUFA) have been proposed to modulate inflammation and immunity[311]. A clinical trial of formula feeds with or without supplementation with PUFA in the form of egg phospholipids in preterm neonates showed that the supplemented formula contained 7-fold more arachidonic acid and docosahexanoic acid and reduced the incidence of stage II and III NEC[312]. Recent evidence from an experimental study indicates that the protective effect of long chain PUFA is mediated by modulation of PAF metabolism and endotoxin translocation[313].

1.15.14 Oral Immunoglobulins

A number of reports have been published, which suggest that oral immunoglobulins (IgA and IgG) have an immunoprotective effect on the gastrointestinal mucosa[314,315]. Premature infants have decreased levels of immunoglobulins, especially secretory IgA[316]. A reduction in the incidence of NEC following feeding an oral IgA-IgG preparation was reported as early as 1988[314]. A systemic review on oral immunoglobulin for the prevention of NEC did not show a significant reduction on the incidence of definite NEC[317]. No randomized controlled trials of oral immunoglobulins for the prevention of NEC have been carried out. Current evidence does not support the administration of oral immunoglobulin for the prevention of NEC.

1.15.15 Epidermal Growth Factor (EGF)

Epidermal growth factor is a growth factor that exerts its effects by binding to the EGF receptor. EGF is an important constituent of gastrointestinal secretions and has multiple effects upon gut epithelial cells including cytoprotection, stimulatory effects on

cell proliferation and migration, induction of mucosal enzyme and trefoil peptide expression and inhibitory effects on gastric acid secretion[172]. Preterm neonates with NEC have diminished levels of salivary and serum EGF[318]. The presence of immunoreactive EGF receptors in gut epithelial cells of preterm neonates with NEC raises the possibility of using EGF for prophylaxis or treatment of NEC[319]. In a neonatal rat model of NEC, EGF treatment maintained intestinal integrity at the site of injury by accelerating goblet cell maturation and mucin production and normalizing expression of tight junction proteins[320]. Researchers have already warranted that the clinical use of EGF may be associated with a variety of problems and side effects and that careful selection of patients and evaluation of risk-benefit ratios are necessary[321]. Given the potential for adverse effects and the fact that maturity alone is not a protective factor for NEC the use of any growth factors in preterm neonates warrants extreme caution.

1.15.16 Erythropoietin

The presence of erythropoietin (Epo) in human milk and the expression of Epo receptors on intestinal villous enterocytes of neonates suggest that Epo has a role in growth and development of the gastrointestinal tract[322-324]. Ledbetter[324] administered recombinant Epo for the prevention and treatment of the anemia of prematurity and demonstrated that the rEpo group had a lower incidence of NEC. Akisu [257,325] indicated that rEpo decreased lipid peroxidation but not PAF generation. Although not completely absorbed, Epo acts as a trophic factor in developing rat bowel whether given enterally or parenterally[323]. Current evidence indicates that the protective effect of rEpo may be related to inhibition of NO formation[326].

1.15.17 Other Experimental Agents

A variety of other experimental agents have been studied in search for an effective agent for the prevention of NEC. These include anti-TNF-α[206], PAF receptor antagonists[327], heparin-binding EGF-like growth factor[328], anti-inflammatory cytokines (IL-10)[329], pentoxifylline[330], intestinal trefoil factor 3[331] and glucagon-like peptide 2.

1.16 References

1. Merrill, A.H. & Sweeley, C.C. Biochemistry of Lipids, Lipoproteins and Membranes. in *Sphingolipids: Metabolism and Cell Signalling*, Vol. 12 (eds. Vance, D.E. & Vance, J.E.) 309-339 (Elsevier Science, St Louis, MO, 2002).
2. Tettamanti, G. Ganglioside/glycosphingolipid turnover: new concepts. *Glycoconj J* 20, 301-17 (2004).
3. Chester, M. IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). Nomenclature of glycolipids--recommendations 1997. *European Journal of Biochemistry* 257, 293-298 (1998).
4. Rueda, R., Maldonado, J., Narbona, E. & Gil, A. Neonatal dietary gangliosides. *Early Hum Dev* 53 Suppl, S135-47 (1998).
5. Wang, B. & Brand-Miller, J. The role and potential of sialic acid in human nutrition. *Eur J Clin Nutr* 57, 1351-69 (2003).
6. Saqr, H.E., Pearl, D.K. & Yates, A.J. A review and predictive models of ganglioside uptake by biological membranes. *J Neurochem* 61, 395-411 (1993).
7. Nichols, B. Caveosomes and endocytosis of lipid rafts. *J Cell Sci* 116, 4707-14 (2003).
8. Fantini, J., Maresca, M., Hammache, D., Yahi, N. & Delezay, O. Glycosphingolipid (GSL) microdomains as attachment platforms for host pathogens and their toxins on intestinal epithelial cells: activation of signal transduction pathways and perturbations of intestinal absorption and secretion. *Glycoconj J* 17, 173-9 (2000).
9. Merrill, A.H., Jr. et al. Sphingolipids--the enigmatic lipid class: biochemistry, physiology, and pathophysiology. *Toxicol Appl Pharmacol* 142, 208-25 (1997).
10. Vesper, H. et al. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J Nutr* 129, 1239-50 (1999).
11. Lunn, P.G. Breast-feeding patterns, maternal milk output and lactational infecundity. *J Biosoc Sci* 24, 317-24 (1992).
12. Newburg, D.S. & Chaturvedi, P. Neutral glycolipids of human and bovine milk. *Lipids* 27, 923-7 (1992).
13. Rueda, R., Puente, R., Hueso, P., Maldonado, J. & Gil, A. New data on content and distribution of gangliosides in human milk. *Biol Chem Hoppe Seyler* 376, 723-7 (1995).
14. Schwarzmann, G. Uptake and metabolism of exogenous glycosphingolipids by cultured cells. *Semin Cell Dev Biol* 12, 163-71 (2001).
15. Clarke, J.T. The glycosphingolipids of human plasma lipoproteins. *Can J Biochem* 59, 412-7 (1981).
16. Draper, J.S., Pigott, C., Thomson, J.A. & Andrews, P.W. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. *J Anat* 200, 249-58 (2002).
17. Kakugawa, Y. et al. Up-regulation of plasma membrane-associated ganglioside sialidase (Neu3) in human colon cancer and its involvement in apoptosis suppression. *Proc Natl Acad Sci U S A* 99, 10718-23 (2002).
18. Takamizawa, K., Iwamori, M., Mutai, M. & Nagai, Y. Selective changes in gangliosides of human milk during lactation: a molecular indicator for the period of lactation. *Biochim Biophys Acta* 879, 73-7 (1986).

19. Park, E.J. et al. Diet-induced changes in membrane gangliosides in rat intestinal mucosa, plasma and brain. *J Pediatr Gastroenterol Nutr* 40, 487-95 (2005).
20. Dahiya, R. & Brasitus, T.A. Distribution of glycosphingolipids and ceramide of rat small intestinal mucosa. *Lipids* 21, 112-6 (1986).
21. Forstner, G.G. & Wherrett, J.R. Plasma membrane and mucosal glycosphingolipids in the rat intestine. *Biochim Biophys Acta* 306, 446-59 (1973).
22. Glickman, R.M. & Bouhours, J.F. Characterization, distribution and biosynthesis of the major ganglioside of rat intestinal mucosa. *Biochim Biophys Acta* 424, 17-25 (1976).
23. Bouhours, J.F., Bouhours, D. & Hansson, G.C. Developmental changes of glycosphingolipid composition of epithelia of rat digestive tract. *Adv Lipid Res* 26, 353-72 (1993).
24. Iwamori, M., Shimomura, J., Tsuyuhara, S. & Nagai, Y. Gangliosides of various rat tissues: distribution of ganglio-N-tetraose-containing gangliosides and tissue-characteristic composition of gangliosides. *J Biochem (Tokyo)* 95, 761-70 (1984).
25. Ogawa-Goto, K. & Abe, T. Gangliosides and glycosphingolipids of peripheral nervous system myelins--a minireview. *Neurochem Res* 23, 305-10 (1998).
26. Marconi, S. et al. Expression of gangliosides on glial and neuronal cells in normal and pathological adult human brain. *J Neuroimmunol* 170, 115-21 (2005).
27. Yates, A.J. Gangliosides in the nervous system during development and regeneration. *Neurochem Pathol* 5, 309-29 (1986).
28. Sela, B.A., Konat, G. & Offner, H. Elevated ganglioside concentration in serum and peripheral blood lymphocytes from multiple sclerosis patients in remission. *J Neurol Sci* 54, 143-8 (1982).
29. Wiels, J., Mangeney, M., Tetaud, C. & Tursz, T. Sequential shifts in the three major glycosphingolipid series are associated with B cell differentiation. *Int Immunol* 3, 1289-300 (1991).
30. Dyatlovitskaya, E.V., Zablotskaya, A.E., Azizov, Y.M. & Bergelson, L.D. Gangliosides of calf thymus and of normal and leukemic bovine lymphocytes. *Eur J Biochem* 110, 475-83 (1980).
31. Karlsson, A. et al. Different glycosphingolipid composition in human neutrophil subcellular compartments. *Glycoconj J* 18, 231-43 (2001).
32. Yohe, H.C. & Ryan, J.L. Ganglioside expression in macrophages from endotoxin responder and nonresponder mice. *J Immunol* 137, 3921-7 (1986).
33. Bode, L., Beermann, C., Mank, M., Kohn, G. & Boehm, G. human and bovine milk gangliosides differ in their fatty acid composition. *J Nutr* 134, 3016-20 (2004).
34. Pan, X.L. & Izumi, T. Variation of the ganglioside compositions of human milk, cow's milk and infant formulas. *Early Hum Dev* 57, 25-31 (2000).
35. Fredman, P., Hedberg, K. & Brezicka, T. Gangliosides as therapeutic targets for cancer. *BioDrugs* 17, 155-67 (2003).

36. Liu, J.J., Nilsson, A. & Duan, R.D. In vitro effects of fat, FA, and cholesterol on sphingomyelin hydrolysis induced by rat intestinal alkaline sphingomyelinase. *Lipids* 37, 469-74 (2002).
37. Schmelz, E.M., Crall, K.J., Larocque, R., Dillehay, D.L. & Merrill, A.H., Jr. Uptake and metabolism of sphingolipids in isolated intestinal loops of mice. *J Nutr* 124, 702-12 (1994).
38. Sugawara, T., Kinoshita, M., Ohnishi, M., Nagata, J. & Saito, M. Digestion of maize sphingolipids in rats and uptake of sphingadienine by Caco-2 cells. *J Nutr* 133, 2777-82 (2003).
39. Lillienau, J., Cheng, Y., Nilsson, A. & Duan, R.D. Development of intestinal alkaline sphingomyelinase in rat fetus and newborn rat. *Lipids* 38, 545-9 (2003).
40. Nyberg, L. et al. Digestion of ceramide by human milk bile salt-stimulated lipase. *J Pediatr Gastroenterol Nutr* 27, 560-7 (1998).
41. Nilsson, A. & Duan, R.D. Absorption and lipoprotein transport of sphingomyelin. *J Lipid Res* 47, 154-71 (2006).
42. Pope-Delatorre, H., Das, S. & Irwin, L.N. Uptake of [3H]-gangliosides by an intestinal protozoan, Giardia lamblia. *Parasitol Res* 96, 102-6 (2005).
43. Schengrund, C.L. & Mummert, C.M. Exogenous gangliosides. How do they cross the blood-brain barrier and how do they inhibit cell proliferation. *Ann N Y Acad Sci* 845, 278-84 (1998).
44. Attrill, H. et al. Siglec-7 undergoes a major conformational change when complexed with the alpha(2,8)-disialylganglioside GT1b. *J Biol Chem* 281, 32774-83 (2006).
45. Lalazar, G., Preston, S., Zigmond, E., Ben Yaacov, A. & Ilan, Y. Glycolipids as immune modulatory tools. *Mini Rev Med Chem* 6, 1249-53 (2006).
46. Milis, D.G., Moore, M.K., Atshaves, B.P., Schroeder, F. & Jefferson, J.R. Sterol carrier protein-2 expression alters sphingolipid metabolism in transfected mouse L-cell fibroblasts. *Mol Cell Biochem* 283, 57-66 (2006).
47. Raggers, R.J., van Helvoort, A., Evers, R. & van Meer, G. The human multidrug resistance protein MRP1 translocates sphingolipid analogs across the plasma membrane. *J Cell Sci* 112 (Pt 3), 415-22 (1999).
48. Taylor, T.M., Davidson, P.M., Bruce, B.D. & Weiss, J. Liposomal nanocapsules in food science and agriculture. *Crit Rev Food Sci Nutr* 45, 587-605 (2005).
49. Rao, C.S., Lin, X., Pike, H.M., Molotkovsky, J.G. & Brown, R.E. Glycolipid transfer protein mediated transfer of glycosphingolipids between membranes: a model for action based on kinetic and thermodynamic analyses. *Biochemistry* 43, 13805-15 (2004).
50. Pagano, R.E. Lipid traffic in eukaryotic cells: mechanisms for intracellular transport and organelle-specific enrichment of lipids. *Curr Opin Cell Biol* 2, 652-63 (1990).
51. Malinina, L. et al. The liganding of glycolipid transfer protein is controlled by glycolipid acyl structure. *PLoS Biol* 4, e362 (2006).
52. Maccioni, H.J., Giraudo, C.G. & Daniotti, J.L. Understanding the stepwise synthesis of glycolipids. *Neurochem Res* 27, 629-36 (2002).
53. Tettamanti, G., Bassi, R., Viani, P. & Riboni, L. Salvage pathways in glycosphingolipid metabolism. *Biochimie* 85, 423-37 (2003).

54. Ozkara, H.A. Recent advances in the biochemistry and genetics of sphingolipidoses. *Brain Dev* 26, 497-505 (2004).
55. Chen, H.Y., Challa, A.K. & Varki, A. 9-O-acetylation of exogenously added ganglioside GD3. The GD3 molecule induces its own O-acetylation machinery. *J Biol Chem* 281, 7825-33 (2006).
56. Riboni, L., Bassi, R., Prinetti, A., Viani, P. & Tettamanti, G. Predominance of the acylation route in the metabolic processing of exogenous sphingosine in neural and extraneural cells in culture. *Biochem J* 338 (Pt 1), 147-51 (1999).
57. Sofer, A., Schwarzmann, G. & Futerman, A.H. The internalization of a short acyl chain analogue of ganglioside GM1 in polarized neurons. *J Cell Sci* 109 (Pt 8), 2111-9 (1996).
58. Ledeen, R.W. Biosynthesis, Metabolism and Biological Effects of Gangliosides. in *Neurobiology of Glycoconjugates* (eds. Margolis, R.U. & Margolis, R.K.) 43-83 (Plenum Press, New York, 1989).
59. Hakomori, S. Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. *J Biol Chem* 265, 18713-6 (1990).
60. Holmgren, J., Lindblad, M., Fredman, P., Svennerholm, L. & Myrvold, H. Comparison of receptors for cholera and Escherichia coli enterotoxins in human intestine. *Gastroenterology* 89, 27-35 (1985).
61. Kyogashima, M., Ginsburg, V. & Krivan, H.C. Escherichia coli K99 binds to N-glycolylsialoparagloboside and N-glycolyl-GM3 found in piglet small intestine. *Arch Biochem Biophys* 270, 391-7 (1989).
62. Laegreid, A. & Kolsto Otnaess, A.B. Trace amounts of ganglioside GM1 in human milk inhibit enterotoxins from Vibrio cholerae and Escherichia coli. *Life Sci* 40, 55-62 (1987).
63. Rolsma, M.D., Kuhlenschmidt, T.B., Gelberg, H.B. & Kuhlenschmidt, M.S. Structure and function of a ganglioside receptor for porcine rotavirus. *J Virol* 72, 9079-91 (1998).
64. Suh, M., Belosevic, M. & Clandinin, M.T. Dietary lipids containing gangliosides reduce Giardia muris infection in vivo and survival of Giardia lamblia trophozoites in vitro. *Parasitology* 128, 595-602 (2004).
65. Gopalakrishna, P., Rangaraj, N. & Pande, G. Cholesterol alters the interaction of glycosphingolipid GM3 with alpha5beta1 integrin and increases integrin-mediated cell adhesion to fibronectin. *Exp Cell Res* 300, 43-53 (2004).
66. Hakomori, S. Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. *Cancer Res* 56, 5309-18 (1996).
67. Kazarian, T., Jabbar, A.A., Wen, F.Q., Patel, D.A. & Valentino, L.A. Gangliosides regulate tumor cell adhesion to collagen. *Clin Exp Metastasis* 20, 311-9 (2003).
68. Mendez-Otero, R. & Santiago, M.F. Functional role of a specific ganglioside in neuronal migration and neurite outgrowth. *Braz J Med Biol Res* 36, 1003-13 (2003).
69. Yang, Z., Zhao, Z. & Panjwani, N. Gangliosides of migrating and nonmigrating corneal epithelium in organ and cell culture. *Invest Ophthalmol Vis Sci* 37, 501-10 (1996).

70. Taieb, N., Yahi, N. & Fantini, J. Rafts and related glycosphingolipid-enriched microdomains in the intestinal epithelium: bacterial targets linked to nutrient absorption. *Adv Drug Deliv Rev* 56, 779-94 (2004).
71. Allende, M.L. & Proia, R.L. Lubricating cell signaling pathways with gangliosides. *Curr Opin Struct Biol* 12, 587-92 (2002).
72. Bathori, G., Cervenak, L. & Karadi, I. Caveolae--an alternative endocytotic pathway for targeted drug delivery. *Crit Rev Ther Drug Carrier Syst* 21, 67-95 (2004).
73. Abraham, S.N., Duncan, M.J., Li, G. & Zaas, D. Bacterial penetration of the mucosal barrier by targeting lipid rafts. *J Investig Med* 53, 318-21 (2005).
74. Lucero, H.A. & Robbins, P.W. Lipid rafts-protein association and the regulation of protein activity. *Arch Biochem Biophys* 426, 208-24 (2004).
75. Chikani, G., Zhu, W. & Smart, E.J. Lipids: potential regulators of nitric oxide generation. *Am J Physiol Endocrinol Metab* 287, E386-9 (2004).
76. Delorme, C. et al. Glycosphingolipid binding specificities of rotavirus: identification of a sialic acid-binding epitope. *J Virol* 75, 2276-87 (2001).
77. Fujiwara, S., Hashiba, H., Hirota, T. & Forstner, J.F. Inhibition of the binding of enterotoxigenic Escherichia coli Pb176 to human intestinal epithelial cell line HCT-8 by an extracellular protein fraction containing BIF of Bifidobacterium longum SBT2928: suggestive evidence of blocking of the binding receptor gangliotetraosylceramide on the cell surface. *Int J Food Microbiol* 67, 97-106 (2001).
78. Kowalski, M.P. & Pier, G.B. Localization of cystic fibrosis transmembrane conductance regulator to lipid rafts of epithelial cells is required for Pseudomonas aeruginosa-induced cellular activation. *J Immunol* 172, 418-25 (2004).
79. Slomiany, B.L. & Slomiany, A. Mechanism of Helicobacter pylori pathogenesis: focus on mucus. *J Clin Gastroenterol* 14 Suppl 1, S114-21 (1992).
80. Tsai, B. et al. Gangliosides are receptors for murine polyoma virus and SV40. *Embo J* 22, 4346-55 (2003).
81. Tsukamoto, K. et al. Binding of Clostridium botulinum type C and D neurotoxins to ganglioside and phospholipid. Novel insights into the receptor for clostridial neurotoxins. *J Biol Chem* 280, 35164-71 (2005).
82. Wimer-Mackin, S., Holmes, R.K., Wolf, A.A., Lencer, W.I. & Jobling, M.G. Characterization of receptor-mediated signal transduction by Escherichia coli type IIa heat-labile enterotoxin in the polarized human intestinal cell line T84. *Infect Immun* 69, 7205-12 (2001).
83. Miljan, E.A. & Bremer, E.G. Regulation of growth factor receptors by gangliosides. *Sci STKE* 2002, RE15 (2002).
84. Yates, A.J. & Rampersaud, A. Sphingolipids as receptor modulators. An overview. *Ann N Y Acad Sci* 845, 57-71 (1998).
85. O'Hanlon, G.M., Hirst, T.R. & Willison, H.J. Ganglioside GM1 binding toxins and human neuropathy-associated IgM antibodies differentially promote neuritogenesis in a PC12 assay. *Neurosci Res* 47, 383-90 (2003).
86. Colell, A., Morales, A., Fernandez-Checa, J.C. & Garcia-Ruiz, C. Ceramide generated by acidic sphingomyelinase contributes to tumor necrosis factor-

alpha-mediated apoptosis in human colon HT-29 cells through glycosphingolipids formation. Possible role of ganglioside GD3. *FEBS Lett* 526, 135-41 (2002).

87. Morales, A., Colell, A., Mari, M., Garcia-Ruiz, C. & Fernandez-Checa, J.C. Glycosphingolipids and mitochondria: role in apoptosis and disease. *Glycoconj J* 20, 579-88 (2004).
88. Wang, J.H. et al. [Ganglioside GD3 enhances the anti-tumor effect of doxorubicin on hepatoma cells]. *Zhonghua Gan Zang Bing Za Zhi* 14, 710-1 (2006).
89. Kniep, B. et al. 9-O-acetyl GD3 protects tumor cells from apoptosis. *Int J Cancer* 119, 67-73 (2006).
90. Ledeen, R.W. & Wu, G. Ganglioside function in calcium homeostasis and signaling. *Neurochem Res* 27, 637-47 (2002).
91. Wu, G., Lu, Z.H. & Ledeen, R.W. GM1 ganglioside in the nuclear membrane modulates nuclear calcium homeostasis during neurite outgrowth. *J Neurochem* 65, 1419-22 (1995).
92. Milani, D. et al. Excitatory amino acid-induced alterations of cytoplasmic free Ca2+ in individual cerebellar granule neurons: role in neurotoxicity. *J Neurosci Res* 28, 434-41 (1991).
93. Krajnc, D., Wemlinger, T.A., Neff, N.H. & Hadjiconstantinou, M. Neonatal hypoxia: early neurotransmitter responses and the consequences of treatment with GM1 ganglioside. *J Pharmacol Exp Ther* 271, 1299-305 (1994).
94. Lodovici, M. et al. Effects of GM1 ganglioside on cardiac function following experimental hypoxia-reoxygenation. *Eur J Pharmacol* 243, 255-63 (1993).
95. Ando, S. et al. Gangliosides and sialylcholesterol as modulators of synaptic functions. *Ann N Y Acad Sci* 845, 232-9 (1998).
96. Fukunaga, K., Miyamoto, E. & Soderling, T.R. Regulation of Ca2+/calmodulin-dependent protein kinase II by brain gangliosides. *J Neurochem* 54, 103-9 (1990).
97. Bulotta, S. et al. Endothelial nitric oxide synthase is segregated from caveolin-1 and localizes to the leading edge of migrating cells. *Exp Cell Res* 312, 877-89 (2006).
98. Marengo, F.D., Wang, S.Y., Wang, B. & Langer, G.A. Dependence of cardiac cell Ca2+ permeability on sialic acid-containing sarcolemmal gangliosides. *J Mol Cell Cardiol* 30, 127-37 (1998).
99. Muthing, J., Maurer, U. & Weber-Schurholz, S. Glycosphingolipids of skeletal muscle: II. Modulation of Ca2(+)-flux in triad membranes by gangliosides. *Carbohydr Res* 307, 147-57 (1998).
100. Caldwell, S. et al. Mechanisms of ganglioside inhibition of APC function. *J Immunol* 171, 1676-83 (2003).
101. Irani, D.N., Lin, K.I. & Griffin, D.E. Brain-derived gangliosides regulate the cytokine production and proliferation of activated T cells. *J Immunol* 157, 4333-40 (1996).
102. Crespo, F.A., Sun, X., Cripps, J.G. & Fernandez-Botran, R. The immunoregulatory effects of gangliosides involve immune deviation favoring type-2 T cell responses. *J Leukoc Biol* 79, 586-95 (2006).

103. Vazquez, E., Gil, A. & Rueda, R. Dietary gangliosides positively modulate the percentages of Th1 and Th2 lymphocyte subsets in small intestine of mice at weaning. *Biofactors* 15, 1-9 (2001).
104. Park, E.J. et al. Dietary ganglioside decreases cholesterol content, caveolin expression and inflammatory mediators in rat intestinal microdomains. *Glycobiology* 15, 935-42 (2005).
105. Raas-Rothschild, A., Pankova-Kholmyansky, I., Kacher, Y. & Futerman, A.H. Glycosphingolipidoses: beyond the enzymatic defect. *Glycoconj J* 21, 295-304 (2004).
106. Aerts, J.M., Hollak, C., Boot, R. & Groener, A. Biochemistry of glycosphingolipid storage disorders: implications for therapeutic intervention. *Philos Trans R Soc Lond B Biol Sci* 358, 905-14 (2003).
107. Mocchetti, I. Exogenous gangliosides, neuronal plasticity and repair, and the neurotrophins. *Cell Mol Life Sci* 62, 2283-94 (2005).
108. Purpura, D.P. & Suzuki, K. Distortion of neuronal geometry and formation of aberrant synapses in neuronal storage disease. *Brain Res* 116, 1-21 (1976).
109. Ceccarelli, B., Aporti, F. & Finesso, M. Effects of brain gangliosides on functional recovery in experimental regeneration and reinnervation. *Adv Exp Med Biol* 71, 275-93 (1976).
110. Schneider, J.S. et al. Parkinson's disease: improved function with GM1 ganglioside treatment in a randomized placebo-controlled study. *Neurology* 50, 1630-6 (1998).
111. Fong, T.G., Neff, N.H. & Hadjiconstantinou, M. GM1 ganglioside improves spatial learning and memory of aged rats. *Behav Brain Res* 85, 203-11 (1997).
112. Sofroniew, M.V., Pearson, R.C., Cuello, A.C., Tagari, P.C. & Stephens, P.H. Parenterally administered GM1 ganglioside prevents retrograde degeneration of cholinergic cells of the rat basal forebrain. *Brain Res* 398, 393-6 (1986).
113. Yanagisawa, K. GM1 ganglioside and the seeding of amyloid in Alzheimer's disease: endogenous seed for Alzheimer amyloid. *Neuroscientist* 11, 250-60 (2005).
114. Leon, A. et al. Hypoxic-ischemic damage and the neuroprotective effects of GM1 ganglioside. *Stroke* 21, III95-7 (1990).
115. Manev, H., Favaron, M., Vicini, S. & Guidotti, A. Ganglioside-mediated protection from glutamate-induced neuronal death. *Acta Neurobiol Exp (Wars)* 50, 475-88 (1990).
116. Chinnock, P. & Roberts, I. Gangliosides for acute spinal cord injury. *Cochrane Database Syst Rev*, CD004444 (2005).
117. Rapport, M.M. Implications of altered brain ganglioside profiles in amyotrophic lateral sclerosis (ALS). *Acta Neurobiol Exp (Wars)* 50, 505-13 (1990).
118. Faries, M.B. & Morton, D.L. Therapeutic vaccines for melanoma: current status. *BioDrugs* 19, 247-60 (2005).
119. Paris, R. et al. Ganglioside GD3 sensitizes human hepatoma cells to cancer therapy. *J Biol Chem* 277, 49870-6 (2002).
120. De Libero, G. & Mori, L. Self glycosphingolipids: new antigens recognized by autoreactive T lymphocytes. *News Physiol Sci* 18, 71-6 (2003).

121. Misasi, R. et al. Gangliosides and autoimmune diabetes. *Diabetes Metab Rev* 13, 163-79 (1997).
122. Monteiro de Castro, G. et al. Th1 and Th2 cytokine immunomodulation by gangliosides in experimental autoimmune encephalomyelitis. *Cytokine* 26, 155-63 (2004).
123. Gomez-Nicola, D., Doncel-Perez, E. & Nieto-Sampedro, M. Regulation by GD3 of the proinflammatory response of microglia mediated by interleukin-15. *J Neurosci Res* 83, 754-62 (2006).
124. Bobryshev, Y.V. et al. Expression of GM3 synthase in human atherosclerotic lesions. *Atherosclerosis* 184, 63-71 (2006).
125. Lin, P.W. & Stoll, B.J. Necrotising enterocolitis. *Lancet* 368, 1271-83 (2006).
126. Lee, J.S. & Polin, R.A. Treatment and prevention of necrotizing enterocolitis. *Semin Neonatol* 8, 449-59 (2003).
127. Updegrove, K. Necrotizing enterocolitis: the evidence for use of human milk in prevention and treatment. *J Hum Lact* 20, 335-9 (2004).
128. Martin, C.R. & Walker, W.A. Intestinal immune defences and the inflammatory response in necrotising enterocolitis. *Semin Fetal Neonatal Med* 11, 369-77 (2006).
129. Henry, M.C. & Lawrence Moss, R. Surgical therapy for necrotizing enterocolitis: bringing evidence to the bedside. *Semin Pediatr Surg* 14, 181-90 (2005).
130. Holman, R.C., Stoll, B.J., Clarke, M.J. & Glass, R.I. The epidemiology of necrotizing enterocolitis infant mortality in the United States. *Am J Public Health* 87, 2026-31 (1997).
131. Israel, E.J. & Morera, C. *Necrotizing Enterocolitis*, 688-691 (Elsevier Science, Cambridge, 2004).
132. Patole, S. Prevention of necrotising enterocolitis: year 2004 and beyond. *J Matern Fetal Neonatal Med* 17, 69-80 (2005).
133. Yost, C.C. Neonatal necrotizing enterocolitis: diagnosis, management, and pathogenesis. *J Infus Nurs* 28, 130-4 (2005).
134. Horton, K.K. Pathophysiology and current management of necrotizing enterocolitis. *Neonatal Netw* 24, 37-46 (2005).
135. Kafetzis, D.A., Skevaki, C. & Costalos, C. Neonatal necrotizing enterocolitis: an overview. *Curr Opin Infect Dis* 16, 349-55 (2003).
136. Pourcyrous, M., Korones, S.B., Yang, W., Boulden, T.F. & Bada, H.S. C-reactive protein in the diagnosis, management, and prognosis of neonatal necrotizing enterocolitis. *Pediatrics* 116, 1064-9 (2005).
137. Sangild, P.T. et al. Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs. *Gastroenterology* 130, 1776-92 (2006).
138. Bell, M.J. et al. Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging. *Ann Surg* 187, 1-7 (1978).
139. Edelson, M.B., Bagwell, C.E. & Rozycki, H.J. Circulating pro- and counterinflammatory cytokine levels and severity in necrotizing enterocolitis. *Pediatrics* 103, 766-71 (1999).

140. Edelson, M.B. et al. Plasma intestinal fatty acid binding protein in neonates with necrotizing enterocolitis: a pilot study. *J Pediatr Surg* 34, 1453-7 (1999).
141. Guthmann, F. et al. Plasma concentration of intestinal- and liver-FABP in neonates suffering from necrotizing enterocolitis and in healthy preterm neonates. *Mol Cell Biochem* 239, 227-34 (2002).
142. Kim, W.Y. et al. Sonographic evaluation of neonates with early-stage necrotizing enterocolitis. *Pediatr Radiol* 35, 1056-61 (2005).
143. Treszl, A., Tulassay, T. & Vasarhelyi, B. Genetic basis for necrotizing enterocolitis--risk factors and their relations to genetic polymorphisms. *Front Biosci* 11, 570-80 (2006).
144. Caplan, M.S. *Pathophysiology and Prevention of Neonatal Necrotizing Enterocolitis*, 1169-1172 (Saunders, St Louis, 2003).
145. Hackam, D.J., Upperman, J.S., Grishin, A. & Ford, H.R. Disordered enterocyte signaling and intestinal barrier dysfunction in the pathogenesis of necrotizing enterocolitis. *Semin Pediatr Surg* 14, 49-57 (2005).
146. Jilling, T., Lu, J., Jackson, M. & Caplan, M.S. Intestinal epithelial apoptosis initiates gross bowel necrosis in an experimental rat model of neonatal necrotizing enterocolitis. *Pediatr Res* 55, 622-9 (2004).
147. Neu, J., Chen, M. & Beierle, E. Intestinal innate immunity: how does it relate to the pathogenesis of necrotizing enterocolitis. *Semin Pediatr Surg* 14, 137-44 (2005).
148. Hsueh, W. et al. Neonatal necrotizing enterocolitis: clinical considerations and pathogenetic concepts. *Pediatr Dev Pathol* 6, 6-23 (2003).
149. Warner, B.W. & Warner, B.B. Role of epidermal growth factor in the pathogenesis of neonatal necrotizing enterocolitis. *Semin Pediatr Surg* 14, 175-80 (2005).
150. Berseth, C.L. Gastrointestinal motility in the neonate. *Clin Perinatol* 23, 179-90 (1996).
151. Sase, M. et al. Ontogeny of fetal rabbit upper gastrointestinal motility. *J Surg Res* 101, 68-72 (2001).
152. Sase, M. et al. Ontogeny of gastric emptying patterns in the human fetus. *J Matern Fetal Neonatal Med* 17, 213-7 (2005).
153. Berseth, C.L. Gut motility and the pathogenesis of necrotizing enterocolitis. *Clin Perinatol* 21, 263-70 (1994).
154. Berseth, C.L. & McCoy, H.H. Birth asphyxia alters neonatal intestinal motility in term neonates. *Pediatrics* 90, 669-73 (1992).
155. Ittmann, P.I., Amarnath, R. & Berseth, C.L. Maturation of antroduodenal motor activity in preterm and term infants. *Dig Dis Sci* 37, 14-9 (1992).
156. Lebenthal, A. & Lebenthal, E. The ontogeny of the small intestinal epithelium. *JPEN J Parenter Enteral Nutr* 23, S3-6 (1999).
157. Lin, J. Too much short chain fatty acids cause neonatal necrotizing enterocolitis. *Med Hypotheses* 62, 291-3 (2004).
158. Lebenthal, E. & Lee, P.C. Development of functional responses in human exocrine pancreas. *Pediatrics* 66, 556-60 (1980).
159. Nusrat, A. et al. Tight junctions are membrane microdomains. *J Cell Sci* 113 (Pt 10), 1771-81 (2000).

160. Hecht, G. Innate mechanisms of epithelial host defense: spotlight on intestine. *Am J Physiol* 277, C351-8 (1999).
161. Buisine, M.P. et al. Mucin gene expression in human embryonic and fetal intestine. *Gut* 43, 519-24 (1998).
162. Israel, E.J. Neonatal necrotizing enterocolitis, a disease of the immature intestinal mucosal barrier. *Acta Paediatr Suppl* 396, 27-32 (1994).
163. Levy, O. Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes. *J Leukoc Biol* 76, 909-25 (2004).
164. Otte, J.M., Kiehne, K. & Herzig, K.H. Antimicrobial peptides in innate immunity of the human intestine. *J Gastroenterol* 38, 717-26 (2003).
165. Chen, H. et al. Recent advances in the research and development of human defensins. *Peptides* 27, 931-40 (2006).
166. Eckmann, L. Defence molecules in intestinal innate immunity against bacterial infections. *Curr Opin Gastroenterol* 21, 147-51 (2005).
167. Lencer, W.I. et al. Induction of epithelial chloride secretion by channel-forming cryptdins 2 and 3. *Proc Natl Acad Sci U S A* 94, 8585-9 (1997).
168. Playford, R.J. et al. The epidermal growth factor receptor (EGF-R) is present on the basolateral, but not the apical, surface of enterocytes in the human gastrointestinal tract. *Gut* 39, 262-6 (1996).
169. Buchmiller, T.L. et al. Effect of transamniotic administration of epidermal growth factor on fetal rabbit small intestinal nutrient transport and disaccharidase development. *J Pediatr Surg* 28, 1239-44 (1993).
170. Hirai, C. et al. Trophic effect of multiple growth factors in amniotic fluid or human milk on cultured human fetal small intestinal cells. *J Pediatr Gastroenterol Nutr* 34, 524-8 (2002).
171. Helmrath, M.A., Shin, C.E., Fox, J.W., Erwin, C.R. & Warner, B.W. Epidermal growth factor in saliva and serum of infants with necrotising enterocolitis. *Lancet* 351, 266-7 (1998).
172. Wong, W.M. & Wright, N.A. Epidermal growth factor, epidermal growth factor receptors, intestinal growth, and adaptation. *JPEN J Parenter Enteral Nutr* 23, S83-8 (1999).
173. Koldovsky, O., Sunshine, P. & Kretchmer, N. Cellular migration of intestinal epithelia in suckling and weaned rats. *Nature* 212, 1389-90 (1966).
174. Hoffmann, W. Trefoil factors TFF (trefoil factor family) peptide-triggered signals promoting mucosal restitution. *Cell Mol Life Sci* 62, 2932-8 (2005).
175. Lin, J. et al. Ontogeny and prenatal expression of trefoil factor 3/ITF in the human intestine. *Early Hum Dev* 71, 103-9 (2003).
176. Nie, S.N., Sun, H.C., Wu, X.H. & Qian, X.M. [Role of trefoil peptides in modulation of gastric adaptation to stress]. *Zhongguo Wei Zhong Bing Ji Jiu Yi Xue* 17, 302-6 (2005).
177. Vieten, D., Corfield, A., Carroll, D., Ramani, P. & Spicer, R. Impaired mucosal regeneration in neonatal necrotising enterocolitis. *Pediatr Surg Int* 21, 153-60 (2005).
178. Vieten, D., Corfield, A., Ramani, P. & Spicer, R. Proliferative response in necrotising enterocolitis is insufficient to prevent disease progression. *Pediatr Surg Int* 22, 50-6 (2006).

179. Donovan, S.M. Role of human milk components in gastrointestinal development: Current knowledge and future needs. *J Pediatr* 149, S49-61 (2006).
180. Calder, P.C. et al. Early nutrition and immunity - progress and perspectives. *Br J Nutr* 96, 774-90 (2006).
181. Jenner, J., Kerst, G., Handgretinger, R. & Muller, I. Increased alpha2,6-sialylation of surface proteins on tolerogenic, immature dendritic cells and regulatory T cells. *Exp Hematol* 34, 1212-8 (2006).
182. Marodi, L. Innate cellular immune responses in newborns. *Clin Immunol* 118, 137-44 (2006).
183. Levy, O. et al. Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. *J Immunol* 173, 4627-34 (2004).
184. Levy, O. et al. The adenosine system selectively inhibits TLR-mediated TNF-alpha production in the human newborn. *J Immunol* 177, 1956-66 (2006).
185. Clapp, D.W. Developmental regulation of the immune system. *Semin Perinatol* 30, 69-72 (2006).
186. Mouzinho, A., Rosenfeld, C.R., Sanchez, P.J. & Risser, R. Revised reference ranges for circulating neutrophils in very-low-birth-weight neonates. *Pediatrics* 94, 76-82 (1994).
187. Yamauchi, A. et al. Rac2-deficient murine macrophages have selective defects in superoxide production and phagocytosis of opsonized particles. *J Immunol* 173, 5971-9 (2004).
188. Yamauchi, A. et al. Rac GTPase isoform-specific regulation of NADPH oxidase and chemotaxis in murine neutrophils in vivo. Role of the C-terminal polybasic domain. *J Biol Chem* 280, 953-64 (2005).
189. Claud, E.C. et al. Developmentally regulated IkappaB expression in intestinal epithelium and susceptibility to flagellin-induced inflammation. *Proc Natl Acad Sci U S A* 101, 7404-8 (2004).
190. Nanthakumar, N.N., Fusunyan, R.D., Sanderson, I. & Walker, W.A. Inflammation in the developing human intestine: A possible pathophysiologic contribution to necrotizing enterocolitis. *Proc Natl Acad Sci U S A* 97, 6043-8 (2000).
191. Dejardin, E. The alternative NF-kappaB pathway from biochemistry to biology: pitfalls and promises for future drug development. *Biochem Pharmacol* 72, 1161-79 (2006).
192. Reber, K.M., Nankervis, C.A. & Nowicki, P.T. Newborn intestinal circulation. Physiology and pathophysiology. *Clin Perinatol* 29, 23-39 (2002).
193. Nowicki, P.T. Ischemia and necrotizing enterocolitis: where, when, and how. *Semin Pediatr Surg* 14, 152-8 (2005).
194. Krediet, T.G. et al. Microbiological factors associated with neonatal necrotizing enterocolitis: protective effect of early antibiotic treatment. *Acta Paediatr* 92, 1180-2 (2003).

195. de la Cochetiere, M.F. et al. Early intestinal bacterial colonization and necrotizing enterocolitis in premature infants: the putative role of Clostridium. *Pediatr Res* 56, 366-70 (2004).
196. Stoll, B.J. & Hansen, N. Infections in VLBW infants: studies from the NICHD Neonatal Research Network. *Semin Perinatol* 27, 293-301 (2003).
197. Caplan, M.S., Simon, D. & Jilling, T. The role of PAF, TLR, and the inflammatory response in neonatal necrotizing enterocolitis. *Semin Pediatr Surg* 14, 145-51 (2005).
198. Hooper, L.V. & Gordon, J.I. Commensal host-bacterial relationships in the gut. *Science* 292, 1115-8 (2001).
199. Collier-Hyams, L.S. & Neish, A.S. Innate immune relationship between commensal flora and the mammalian intestinal epithelium. *Cell Mol Life Sci* 62, 1339-48 (2005).
200. Neish, A.S. Bacterial inhibition of eukaryotic pro-inflammatory pathways. *Immunol Res* 29, 175-86 (2004).
201. Wallace, T.D., Bradley, S., Buckley, N.D. & Green-Johnson, J.M. Interactions of lactic acid bacteria with human intestinal epithelial cells: effects on cytokine production. *J Food Prot* 66, 466-72 (2003).
202. Lin, H.C. et al. Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants. *Pediatrics* 115, 1-4 (2005).
203. Lin, H.C., Su, B.H. & Oh, W. Oral probiotics prevent necrotizing enterocolitis. *J Pediatr* 148, 849; author reply 850 (2006).
204. Ahrens, P. et al. Mutations of genes involved in the innate immune system as predictors of sepsis in very low birth weight infants. *Pediatr Res* 55, 652-6 (2004).
205. Szebeni, B. et al. Genetic polymorphisms of CD14, toll-like receptor 4, and caspase-recruitment domain 15 are not associated with necrotizing enterocolitis in very low birth weight infants. *J Pediatr Gastroenterol Nutr* 42, 27-31 (2006).
206. Halpern, M.D. et al. Reduction of experimental necrotizing enterocolitis with anti-TNF-alpha. *Am J Physiol Gastrointest Liver Physiol* 290, G757-64 (2006).
207. Seitz, G. et al. Protective effect of tumor necrosis factor alpha antibody on experimental necrotizing enterocolitis in the rat. *J Pediatr Surg* 40, 1440-5 (2005).
208. Treszl, A. et al. Genetic variants of the tumour necrosis factor-alpha promoter gene do not influence the development of necrotizing enterocolitis. *Acta Paediatr* 90, 1182-5 (2001).
209. Di Lorenzo, M. & Krantis, A. Altered nitric oxide production in the premature gut may increase susceptibility to intestinal damage in necrotizing enterocolitis. *J Pediatr Surg* 36, 700-5 (2001).
210. Upperman, J.S. et al. Mechanisms of nitric oxide-mediated intestinal barrier failure in necrotizing enterocolitis. *Semin Pediatr Surg* 14, 159-66 (2005).
211. Nowicki, P.T. et al. Endothelial nitric oxide synthase in human intestine resected for necrotizing enterocolitis. *J Pediatr* 150, 40-5 (2007).
212. Ford, H.R. Mechanism of nitric oxide-mediated intestinal barrier failure: insight into the pathogenesis of necrotizing enterocolitis. *J Pediatr Surg* 41, 294-9 (2006).

213. Tarnawski, A.S. & Jones, M.K. The role of epidermal growth factor (EGF) and its receptor in mucosal protection, adaptation to injury, and ulcer healing: involvement of EGF-R signal transduction pathways. *J Clin Gastroenterol* 27 Suppl 1, S12-20 (1998).
214. Nowicki, P.T. et al. Endothelin-1 in human intestine resected for necrotizing enterocolitis. *J Pediatr* 146, 805-10 (2005).
215. Kim, D.Y. & Camilleri, M. Serotonin: a mediator of the brain-gut connection. *Am J Gastroenterol* 95, 2698-709 (2000).
216. Gershon, M.D. Plasticity in serotonin control mechanisms in the gut. *Curr Opin Pharmacol* 3, 600-7 (2003).
217. Crowell, M.D., Shetzline, M.A., Moses, P.L., Mawe, G.M. & Talley, N.J. Enterochromaffin cells and 5-HT signaling in the pathophysiology of disorders of gastrointestinal function. *Curr Opin Investig Drugs* 5, 55-60 (2004).
218. Stiskal, J.A., Kulin, N., Koren, G., Ho, T. & Ito, S. Neonatal paroxetine withdrawal syndrome. *Arch Dis Child Fetal Neonatal Ed* 84, F134-5 (2001).
219. Cabral, G.A. Lipids as bioeffectors in the immune system. *Life Sci* 77, 1699-710 (2005).
220. Park, J.Y., Pillinger, M.H. & Abramson, S.B. Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases. *Clin Immunol* 119, 229-40 (2006).
221. Mitchell, S. et al. Lipoxins, aspirin-triggered epi-lipoxins, lipoxin stable analogues, and the resolution of inflammation: stimulation of macrophage phagocytosis of apoptotic neutrophils in vivo. *J Am Soc Nephrol* 13, 2497-507 (2002).
222. Ramstedt, U. et al. Lipoxin A-induced inhibition of human natural killer cell cytotoxicity: studies on stereospecificity of inhibition and mode of action. *J Immunol* 138, 266-70 (1987).
223. Halpern, M.D. et al. Up-regulation of IL-18 and IL-12 in the ileum of neonatal rats with necrotizing enterocolitis. *Pediatr Res* 51, 733-9 (2002).
224. Markel, T.A. et al. Cytokines in necrotizing enterocolitis. *Shock* 25, 329-37 (2006).
225. Viscardi, R.M., Lyon, N.H., Sun, C.C., Hebel, J.R. & Hasday, J.D. Inflammatory cytokine mRNAs in surgical specimens of necrotizing enterocolitis and normal newborn intestine. *Pediatr Pathol Lab Med* 17, 547-59 (1997).
226. Barreda, D.R., Hanington, P.C. & Belosevic, M. Regulation of myeloid development and function by colony stimulating factors. *Dev Comp Immunol* 28, 509-54 (2004).
227. Shi, Y. et al. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don't know. *Cell Res* 16, 126-33 (2006).
228. Hamilton, J.A. GM-CSF in inflammation and autoimmunity. *Trends Immunol* 23, 403-8 (2002).
229. de Vries, E.G. et al. A double-blind placebo-controlled study with granulocyte-macrophage colony-stimulating factor during chemotherapy for ovarian carcinoma. *Cancer Res* 51, 116-22 (1991).
230. Baud, V. & Karin, M. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol* 11, 372-7 (2001).

231. Ebach, D.R., Riehl, T.E. & Stenson, W.F. Opposing effects of tumor necrosis factor receptor 1 and 2 in sepsis due to cecal ligation and puncture. *Shock* 23, 311-8 (2005).
232. Pender, S.L. et al. Matrix metalloproteinases in necrotising enterocolitis. *Pediatr Res* 54, 160-4 (2003).
233. Dunne, A. & O'Neill, L.A. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci STKE* 2003, re3 (2003).
234. Kishimoto, T. et al. The molecular biology of interleukin 6 and its receptor. *Ciba Found Symp* 167, 5-16; discussion 16-23 (1992).
235. Tilg, H., Dinarello, C.A. & Mier, J.W. IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. *Immunol Today* 18, 428-32 (1997).
236. Goepfert, A.R. et al. Umbilical cord plasma interleukin-6 concentrations in preterm infants and risk of neonatal morbidity. *Am J Obstet Gynecol* 191, 1375-81 (2004).
237. Mahalingam, S. & Karupiah, G. Chemokines and chemokine receptors in infectious diseases. *Immunol Cell Biol* 77, 469-75 (1999).
238. Mukaida, N. Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation. *Int J Hematol* 72, 391-8 (2000).
239. Mazzucchelli, L. et al. Expression of interleukin-8 gene in inflammatory bowel disease is related to the histological grade of active inflammation. *Am J Pathol* 144, 997-1007 (1994).
240. Kang, B.Y., Kim, E. & Kim, T.S. Regulatory mechanisms and their therapeutic implications of interleukin-12 production in immune cells. *Cell Signal* 17, 665-73 (2005).
241. Plevy, S.E., Gemberling, J.H., Hsu, S., Dorner, A.J. & Smale, S.T. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol Cell Biol* 17, 4572-88 (1997).
242. Muhl, H. & Pfeilschifter, J. Interleukin-18 bioactivity: a novel target for immunopharmacological anti-inflammatory intervention. *Eur J Pharmacol* 500, 63-71 (2004).
243. Gracie, J.A., Robertson, S.E. & McInnes, I.B. Interleukin-18. *J Leukoc Biol* 73, 213-24 (2003).
244. Opal, S.M. & DePalo, V.A. Anti-inflammatory cytokines. *Chest* 117, 1162-72 (2000).
245. Karttunnen, R., Breese, E.J., Walker-Smith, J.A. & MacDonald, T.T. Decreased mucosal interleukin-4 (IL-4) production in gut inflammation. *J Clin Pathol* 47, 1015-8 (1994).
246. Chheda, S., Palkowetz, K.H., Garofalo, R., Rassin, D.K. & Goldman, A.S. Decreased interleukin-10 production by neonatal monocytes and T cells: relationship to decreased production and expression of tumor necrosis factor-alpha and its receptors. *Pediatr Res* 40, 475-83 (1996).
247. Le, T., Leung, L., Carroll, W.L. & Schibler, K.R. Regulation of interleukin-10 gene expression: possible mechanisms accounting for its upregulation and for

maturational differences in its expression by blood mononuclear cells. *Blood* 89, 4112-9 (1997).
248. Jones, C.A. et al. Undetectable interleukin (IL)-10 and persistent IL-8 expression early in hyaline membrane disease: a possible developmental basis for the predisposition to chronic lung inflammation in preterm newborns. *Pediatr Res* 39, 966-75 (1996).
249. Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. & Muller, W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75, 263-74 (1993).
250. Lane, J.S. et al. Interleukin-10 reduces the systemic inflammatory response in a murine model of intestinal ischemia/reperfusion. *Surgery* 122, 288-94 (1997).
251. Pender, S.L. et al. Suppression of T cell-mediated injury in human gut by interleukin 10: role of matrix metalloproteinases. *Gastroenterology* 115, 573-83 (1998).
252. Kling, K.M., Kirby, L., Kwan, K.Y., Kim, F. & McFadden, D.W. Interleukin-10 inhibits inducible nitric oxide synthase in an animal model of necrotizing enterocolitis. *Int J Surg Investig* 1, 337-42 (1999).
253. Granger, D.N. Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. *Am J Physiol* 255, H1269-75 (1988).
254. Qu, X.W., Rozenfeld, R.A., Huang, W., Bulkley, G.B. & Hsueh, W. The role of xanthine oxidase in platelet activating factor induced intestinal injury in the rat. *Gut* 44, 203-11 (1999).
255. Cueva, J.P. & Hsueh, W. Role of oxygen derived free radicals in platelet activating factor induced bowel necrosis. *Gut* 29, 1207-12 (1988).
256. Barlow, B. & Santulli, T.V. Importance of multiple episodes of hypoxia or cold stress on the development of enterocolitis in an animal model. *Surgery* 77, 687-90 (1975).
257. Akisu, M., Tuzun, S., Arslanoglu, S., Yalaz, M. & Kultursay, N. Effect of recombinant human erythropoietin administration on lipid peroxidation and antioxidant enzyme(s) activities in preterm infants. *Acta Med Okayama* 55, 357-62 (2001).
258. Caplan, M.S., Hedlund, E., Hill, N. & MacKendrick, W. The role of endogenous nitric oxide and platelet-activating factor in hypoxia-induced intestinal injury in rats. *Gastroenterology* 106, 346-52 (1994).
259. Chung, D.H. et al. Molecular mechanisms contributing to necrotizing enterocolitis. *Ann Surg* 233, 835-42 (2001).
260. Torimoto, K. et al. Development of multiple necrotizing enteritis induced by a tumor necrosis factor-like cytokine from lipopolysaccharide-stimulated peritoneal macrophages in rats. *Am J Pathol* 137, 1103-11 (1990).
261. Chan, K.L., Ng, S.P., Chan, K.W., Wo, Y.H. & Tam, P.K. Pathogenesis of neonatal necrotizing enterocolitis: a study of the role of intraluminal pressure, age and bacterial concentration. *Pediatr Surg Int* 19, 573-7 (2003).
262. Minekawa, R. et al. Human breast milk suppresses the transcriptional regulation of IL-1beta-induced NF-kappaB signaling in human intestinal cells. *Am J Physiol Cell Physiol* 287, C1404-11 (2004).
263. Crouse, D.T. *Necrotizing Enterocolitis*, 363-373 (Appleton and Lange, Columbus, Ohio, 1993).

264. Moss, R.L. et al. Laparotomy versus peritoneal drainage for necrotizing enterocolitis and perforation. *N Engl J Med* 354, 2225-34 (2006).
265. Pierro, A. The surgical management of necrotising enterocolitis. *Early Hum Dev* 81, 79-85 (2005).
266. Lucas, A. et al. Randomized outcome trial of human milk fortification and developmental outcome in preterm infants. *Am J Clin Nutr* 64, 142-51 (1996).
267. Caplan, M.S., Amer, M. & Jilling, T. The role of human milk in necrotizing enterocolitis. *Adv Exp Med Biol* 503, 83-90 (2002).
268. Hanson, L.A. Human milk and host defence: immediate and long-term effects. *Acta Paediatr Suppl* 88, 42-6 (1999).
269. Akisu, M., Kultursay, N., Ozkayin, N., Coker, I. & Huseyinov, A. Platelet-activating factor levels in term and preterm human milk. *Biol Neonate* 74, 289-93 (1998).
270. Caplan, M., Hsueh, W., Kelly, A. & Donovan, M. Serum PAF acetylhydrolase increases during neonatal maturation. *Prostaglandins* 39, 705-14 (1990).
271. McClure, R.J. & Newell, S.J. Randomised controlled trial of trophic feeding and gut motility. *Arch Dis Child Fetal Neonatal Ed* 80, F54-8 (1999).
272. Buchmiller, T.L. et al. Effect of prenatal dexamethasone administration: fetal rabbit intestinal nutrient uptake and disaccharidase development. *J Surg Res* 57, 274-9 (1994).
273. Israel, E.J., Schiffrin, E.J., Carter, E.A., Freiberg, E. & Walker, W.A. Prevention of necrotizing enterocolitis in the rat with prenatal cortisone. *Gastroenterology* 99, 1333-8 (1990).
274. Bauer, C.R. et al. A decreased incidence of necrotizing enterocolitis after prenatal glucocorticoid therapy. *Pediatrics* 73, 682-8 (1984).
275. Crowley, P., Chalmers, I. & Keirse, M.J. The effects of corticosteroid administration before preterm delivery: an overview of the evidence from controlled trials. *Br J Obstet Gynaecol* 97, 11-25 (1990).
276. Halac, E. et al. Prenatal and postnatal corticosteroid therapy to prevent neonatal necrotizing enterocolitis: a controlled trial. *J Pediatr* 117, 132-8 (1990).
277. Kavelaars, A., Ballieux, R.E. & Heijnen, C.J. Beta-endorphin secretion by human peripheral blood mononuclear cells: regulation by glucocorticoids. *Life Sci* 46, 1233-40 (1990).
278. Bury, R.G. & Tudehope, D. Enteral antibiotics for preventing necrotizing enterocolitis in low birthweight or preterm infants. *Cochrane Database Syst Rev*, CD000405 (2001).
279. Uauy, R.D. et al. Necrotizing enterocolitis in very low birth weight infants: biodemographic and clinical correlates. National Institute of Child Health and Human Development Neonatal Research Network. *J Pediatr* 119, 630-8 (1991).
280. Brown, E.G. & Sweet, A.Y. Preventing necrotizing enterocolitis in neonates. *Jama* 240, 2452-4 (1978).
281. Kamitsuka, M.D., Horton, M.K. & Williams, M.A. The incidence of necrotizing enterocolitis after introducing standardized feeding schedules for infants between 1250 and 2500 grams and less than 35 weeks of gestation. *Pediatrics* 105, 379-84 (2000).

282. Patole, S.K. et al. Benefits of a standardised feeding regimen during a clinical trial in preterm neonates. *Int J Clin Pract* 54, 429-31 (2000).
283. Patole, S.K. & de Klerk, N. Impact of standardised feeding regimens on incidence of neonatal necrotising enterocolitis: a systematic review and meta-analysis of observational studies. *Arch Dis Child Fetal Neonatal Ed* 90, F147-51 (2005).
284. Bell, E.F. & Acarregui, M.J. Restricted versus liberal water intake for preventing morbidity and mortality in preterm infants. *Cochrane Database Syst Rev*, CD000503 (2001).
285. Bourlioux, P., Bouley, C. & Ashwell, M. New aspects of the functionalities of probiotics. *Forum Nutr* 56, 355-6 (2003).
286. Millar, M., Wilks, M. & Costeloe, K. Probiotics for preterm infants? *Arch Dis Child Fetal Neonatal Ed* 88, F354-8 (2003).
287. Dani, C., Biadaioli, R., Bertini, G., Martelli, E. & Rubaltelli, F.F. Probiotics feeding in prevention of urinary tract infection, bacterial sepsis and necrotizing enterocolitis in preterm infants. A prospective double-blind study. *Biol Neonate* 82, 103-8 (2002).
288. Hoyos, A.B. Reduced incidence of necrotizing enterocolitis associated with enteral administration of Lactobacillus acidophilus and Bifidobacterium infantis to neonates in an intensive care unit. *Int J Infect Dis* 3, 197-202 (1999).
289. Bin-Nun, A. et al. Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. *J Pediatr* 147, 192-6 (2005).
290. Ouwehand, A.C., Derrien, M., de Vos, W., Tiihonen, K. & Rautonen, N. Prebiotics and other microbial substrates for gut functionality. *Curr Opin Biotechnol* 16, 212-7 (2005).
291. Knol, J. et al. Increase of faecal bifidobacteria due to dietary oligosaccharides induces a reduction of clinically relevant pathogen germs in the faeces of formula-fed preterm infants. *Acta Paediatr Suppl* 94, 31-3 (2005).
292. Fanaro, S. et al. Galacto-oligosaccharides and long-chain fructo-oligosaccharides as prebiotics in infant formulas: a review. *Acta Paediatr Suppl* 94, 22-6 (2005).
293. Bartholome, A.L., Albin, D.M., Baker, D.H., Holst, J.J. & Tappenden, K.A. Supplementation of total parenteral nutrition with butyrate acutely increases structural aspects of intestinal adaptation after an 80% jejunoileal resection in neonatal piglets. *JPEN J Parenter Enteral Nutr* 28, 210-22; discussion 222-3 (2004).
294. Tsukahara, T., Iwasaki, Y., Nakayama, K. & Ushida, K. Stimulation of butyrate production in the large intestine of weaning piglets by dietary fructooligosaccharides and its influence on the histological variables of the large intestinal mucosa. *J Nutr Sci Vitaminol (Tokyo)* 49, 414-21 (2003).
295. Kanauchi, O. et al. Butyrate from bacterial fermentation of germinated barley foodstuff preserves intestinal barrier function in experimental colitis in the rat model. *J Gastroenterol Hepatol* 14, 880-8 (1999).
296. Mentschel, J. & Claus, R. Increased butyrate formation in the pig colon by feeding raw potato starch leads to a reduction of colonocyte apoptosis and a shift to the stem cell compartment. *Metabolism* 52, 1400-5 (2003).

297. Scheppach, W. & Weiler, F. The butyrate story: old wine in new bottles? *Curr Opin Clin Nutr Metab Care* 7, 563-7 (2004).
298. Rachmilewitz, D. et al. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology* 126, 520-8 (2004).
299. Mackey, D. & McFall, A.J. MAMPs and MIMPs: proposed classifications for inducers of innate immunity. *Mol Microbiol* 61, 1365-71 (2006).
300. Becker, R.M. et al. Reduced serum amino acid concentrations in infants with necrotizing enterocolitis. *J Pediatr* 137, 785-93 (2000).
301. Zamora, S.A. et al. Plasma L-arginine concentrations in premature infants with necrotizing enterocolitis. *J Pediatr* 131, 226-32 (1997).
302. Amin, H.J. et al. Arginine supplementation prevents necrotizing enterocolitis in the premature infant. *J Pediatr* 140, 425-31 (2002).
303. Lass, A., Suessenbacher, A., Wolkart, G., Mayer, B. & Brunner, F. Functional and analytical evidence for scavenging of oxygen radicals by L-arginine. *Mol Pharmacol* 61, 1081-8 (2002).
304. Neu, J. Arginine supplementation and the prevention of necrotizing enterocolitis in very low birth weight infants. *J Pediatr* 140, 389-91 (2002).
305. Shah, P. & Shah, V. Arginine supplementation for prevention of necrotising enterocolitis in preterm infants. *Cochrane Database Syst Rev*, CD004339 (2004).
306. Saugstad, O.D. Update on oxygen radical disease in neonatology. *Curr Opin Obstet Gynecol* 13, 147-53 (2001).
307. Miller, M.J., McNeill, H., Mullane, K.M., Caravella, S.J. & Clark, D.A. SOD prevents damage and attenuates eicosanoid release in a rabbit model of necrotizing enterocolitis. *Am J Physiol* 255, G556-65 (1988).
308. Suresh, G.K., Davis, J.M. & Soll, R.F. Superoxide dismutase for preventing chronic lung disease in mechanically ventilated preterm infants. *Cochrane Database Syst Rev*, CD001968 (2001).
309. Botsford, K.B. et al. Gram-negative bacilli in human milk feedings: quantitation and clinical consequences for premature infants. *J Pediatr* 109, 707-10 (1986).
310. Carrion, V. & Egan, E.A. Prevention of neonatal necrotizing enterocolitis. *J Pediatr Gastroenterol Nutr* 11, 317-23 (1990).
311. Uauy, R., Mena, P. & Rojas, C. Essential fatty acids in early life: structural and functional role. *Proc Nutr Soc* 59, 3-15 (2000).
312. Carlson, S.E., Montalto, M.B., Ponder, D.L., Werkman, S.H. & Korones, S.B. Lower incidence of necrotizing enterocolitis in infants fed a preterm formula with egg phospholipids. *Pediatr Res* 44, 491-8 (1998).
313. Caplan, M.S. & Jilling, T. The role of polyunsaturated fatty acid supplementation in intestinal inflammation and neonatal necrotizing enterocolitis. *Lipids* 36, 1053-7 (2001).
314. Eibl, M.M., Wolf, H.M., Furnkranz, H. & Rosenkranz, A. Prevention of necrotizing enterocolitis in low-birth-weight infants by IgA-IgG feeding. *N Engl J Med* 319, 1-7 (1988).

315. Rubaltelli, F.F., Benini, F. & Sala, M. Prevention of necrotizing enterocolitis in neonates at risk by oral administration of monomeric IgG. *Dev Pharmacol Ther* 17, 138-43 (1991).
316. Fast, C. & Rosegger, H. Necrotizing enterocolitis prophylaxis: oral antibiotics and lyophilized enterobacteria vs oral immunoglobulins. *Acta Paediatr Suppl* 396, 86-90 (1994).
317. Foster, J. & Cole, M. Oral immunoglobulin for preventing necrotizing enterocolitis in preterm and low birth-weight neonates. *Cochrane Database Syst Rev*, CD001816 (2004).
318. Shin, C.E., Falcone, R.A., Jr., Stuart, L., Erwin, C.R. & Warner, B.W. Diminished epidermal growth factor levels in infants with necrotizing enterocolitis. *J Pediatr Surg* 35, 173-6; discussion 177 (2000).
319. Fagbemi, A.O., Wright, N., Lakhoo, K. & Edwards, A.D. Immunoreactive epidermal growth factor receptors are present in gastrointestinal epithelial cells of preterm infants with necrotising enterocolitis. *Early Hum Dev* 65, 1-9 (2001).
320. Clark, J.A. et al. Intestinal barrier failure during experimental necrotizing enterocolitis: protective effect of EGF treatment. *Am J Physiol Gastrointest Liver Physiol* 291, G938-49 (2006).
321. Guglietta, A. & Sullivan, P.B. Clinical applications of epidermal growth factor. *Eur J Gastroenterol Hepatol* 7, 945-50 (1995).
322. Juul, S.E. Erythropoietin in the neonate. *Curr Probl Pediatr* 29, 129-49 (1999).
323. Juul, S.E. et al. Erythropoietin acts as a trophic factor in neonatal rat intestine. *Gut* 49, 182-9 (2001).
324. Ledbetter, D.J. & Juul, S.E. Erythropoietin and the incidence of necrotizing enterocolitis in infants with very low birth weight. *J Pediatr Surg* 35, 178-81; discussion 182 (2000).
325. Akisu, M., Kullahcioglu Girgin, F., Baka, M., Husseyinov, A. & Kultursay, N. The role of recombinant human erythropoietin in lipid peroxidation and platelet-activating factor generation in a rat model of necrotizing enterocolitis. *Eur J Pediatr Surg* 11, 167-72 (2001).
326. Kumral, A. et al. Erythropoietin protects against necrotizing enterocolitis of newborn rats by the inhibiting nitric oxide formation. *Biol Neonate* 84, 325-9 (2003).
327. Caplan, M.S., Hedlund, E., Adler, L., Lickerman, M. & Hsueh, W. The platelet-activating factor receptor antagonist WEB 2170 prevents neonatal necrotizing enterocolitis in rats. *J Pediatr Gastroenterol Nutr* 24, 296-301 (1997).
328. Feng, J., El-Assal, O.N. & Besner, G.E. Heparin-binding EGF-like growth factor (HB-EGF) and necrotizing enterocolitis. *Semin Pediatr Surg* 14, 167-74 (2005).
329. Ozturk, H., Dokucu, A.I., Ogun, C. & Buyukbayram, H. Protective effects of recombinant human interleukin-10 on intestines of hypoxia-induced necrotizing enterocolitis in immature rats. *J Pediatr Surg* 37, 1330-3 (2002).
330. Travadi, J. et al. Pentoxifylline reduces the incidence and severity of necrotizing enterocolitis in a neonatal rat model. *Pediatr Res* 60, 185-9 (2006).

CHAPTER 2 RESEARCH PLAN

2.1 Introduction and Rationale

Gangliosides are amphipathic glycosphingolipids found in human milk and cell membranes of various cells including the brush border and basolateral membrane of enterocytes. Ganglioside GD3 is particularly abundant in colostrum, developing tissues and several tumors. These lipids are known to modulate various important biological processes including cell recognition, adhesion, migration, proliferation, differentiation, signaling and apoptosis. Gangliosides also play an important role in maintaining intestinal barrier function and influence nutrient absorption and the attachment and entry of microorganisms. Human milk gangliosides are essential for mucosal immune system development and function in the early neonatal period when infants are dependent on adequate nutrient supply for rapid growth, development and protection from infections and environmental antigens. Preterm infants are particularly susceptible to infections, ischemic insults and intolerance to rapid advancement of formula feeds, risk factors associated with the development of necrotizing enterocolitis, an inflammatory bowel disease of neonates that impairs gut function. The pathogenesis of necrotizing enterocolitis is elusive due to serious limitations in current disease models. The role of local vasoactive and inflammatory mediators is particularly unclear. Consequently, morbidity and mortality remains high and there is no cure; necrotizing enterocolitis is managed by symptomatic treatment and invasive surgery. Although breast milk is known to promote infant development and reduce risk of infections and necrotizing enterocolitis, limited research has investigated the role of bioactive nutrients from breast milk in the prevention or treatment of necrotizing enterocolitis. The ganglioside composition of cancer cells often resembles that of developing tissues suggesting potential use of intestinal cancer cell lines as models for studying neonatal intestine. Research suggests that gangliosides have anti-inflammatory effects in rat intestine during infection and protect tissues from hypoxic insults. It is not known whether gangliosides have a protective role in infant bowel during infection and hypoxia and whether gangliosides modulate vasoactive, inflammatory signals. Infant formulas and intravenous lipid emulsions do not contain gangliosides. In order to promote development and prevent intestinal conditions such as inflammation, it is

crucial to understand the mechanism and efficiency of ganglioside absorption through both enteral and intravenous routes of delivery. Dietary gangliosides are taken up by rat intestine and appear in intestinal mucosa lipid rafts and plasma, however, the mechanism, efficiency and fate of ganglioside uptake by human enterocytes has not been investigated. This research project will examine the ganglioside composition of differentiated colon cancer cells, ganglioside GD3 absorption by human enterocytes and protective roles of gangliosides in infant bowel during LPS exposure and hypoxia.

2.2 Objectives and Specific Aims

This research project was conducted to evaluate differentiated CaCo-2 cells as a model for neonatal intestine and to investigate enterocyte uptake of ganglioside GD3. The main focus of this study was to determine whether gangliosides protect infant bowel by restoring vasoactive/inflammatory mediator balance during LPS exposure and hypoxia.

The specific aims of this research project are to:

- examine the ganglioside content and composition of undifferentiated and differentiated CaCo-2 cells
- determine the ganglioside concentration range that does not exhibit toxicity to CaCo-2 cells
- examine and compare the time frame, concentration influence and efficiency of apical and basolateral GD3 uptake by CaCo-2 cells
- determine the proportion of GD3 retained, metabolized or transferred across the enterocyte following uptake from the apical or basolateral side of CaCo-2 cell monolayers
- develop an infant bowel model to study necrotizing enterocolitis
- determine whether gangliosides protect infant bowel from necrosis during LPS exposure and hypoxia
- examine the effect of gangliosides on infant bowel production of vasoactive and inflammatory mediators (nitric oxide, endothelin-1,

serotonin, eicosanoids, hydrogen peroxide and cytokines) during LPS exposure and hypoxia

2.3 Hypotheses

The ganglioside composition of differentiated CaCo-2 cells will resemble that of human colostrum and neonatal rat intestine. Ganglioside GD3 uptake from the apical and basolateral side of enterocytes will be fast, efficient and reach a plateau. Route of delivery will influence GD3 uptake efficiency and fate. Incubation with gangliosides will reduce vasoactive and inflammatory signals produced by infant bowel during LPS exposure and hypoxia, thus protecting infant bowel from necrosis and inflammation. It is specifically hypothesized that:

Hypothesis 1: Differentiated CaCo-2 cells will have greater amounts of total gangliosides, GD3 and polar/complex gangliosides than undifferentiated CaCo-2 cells thereby resembling the ganglioside composition of human colostrum and neonatal rat intestine. A decrease in the GM3:GD3 ratio will be observed when CaCo-2 cells are differentiated.

Hypothesis 2: Based on previous in vitro ganglioside experiments with *Giardia* trophozoites and the lipid bound sialic acid content of human colostrum, the ganglioside concentration range that will demonstrate efficacy without toxicity to CaCo-2 cells will be 4-9 μg/ml of cell culture media.

Hypothesis 3: Based on ganglioside GM1 uptake studies with other cell lines, ganglioside GD3 uptake will be time- and concentration- dependent. GD3 uptake will reach a plateau. Based on sphingomyelin digestion and absorption experiments in rodents, apical GD3 uptake will be greater than 40% efficient. Since drugs are generally best delivered to tissues from the circulation, it is expected that basolateral GD3 uptake by CaCo-2 cells will be more efficient than apical GD3 uptake.

Hypothesis 4: Route of delivery will influence GD3 fate. Since Dr. Park demonstrated appearance of dietary GD3 in intestinal mucosa lipid rafts and plasma, it is

Hypothesis 4: Route of delivery will influence GD3 fate. Since Dr. Park demonstrated appearance of dietary GD3 in intestinal mucosa lipid rafts and plasma, it is hypothesized that apical GD3 will be retained, metabolized and transferred across the basolateral membrane. Knowing that GD3 functions in modulating cell signaling pathways, it is hypothesized that basolateral GD3 will be retained and metabolized.

Hypothesis 5: Viable, non-inflamed infant bowel may be collected, transported and cultured following intestinal surgery. Cultured infant bowel will undergo necrosis and produce vasoactive and inflammatory signals in response to enterotoxic *E. coli* LPS and the oxygen chelator sodium thioglycollate.

Hypothesis 6: Incubation with ganglioside reduces necrosis of infant bowel during LPS exposure and hypoxia.

Hypothesis 7: Incubation with ganglioside reduces infant bowel production of nitric oxide, endothelin-1 and serotonin during LPS exposure and hypoxia. Moreover, gangliosides will reduce infant bowel production of eicosanoids, hydrogen peroxide and pro-inflammatory cytokines during LPS exposure and hypoxia.

CHAPTER 3

CHANGE IN GANGLIOSIDE COMPOSITION DURING HUMAN COLON CANCER CELL DIFFERENTIATION

3.1 Introduction

Gangliosides, amphiphilic glycosphingolipids containing sialic acid (N-acetyl neuraminic acid, NANA), are found in plasma membranes of mammalian cells and are biologically important molecules involved in cell differentiation, proliferation, growth, adhesion, migration, signaling and apoptosis[1-3]. In normal physiological processes such as embryogenesis and lactation[4] and in pathological conditions including tumor onset and progression[5], changes in ganglioside composition occur and have been shown to play significant regulatory roles. For example, melanoma cells, embryonic stem cells and human colostrum show an increase in ganglioside content and express more GD3 than normal adult cells and mature human milk[6,7]. Moreover, the GM3 to GD3 ratio increases during development and lactation and decreases during cancer cell differentiation from highly metastatic (poorly differentiated) cancer cells to benign (highly differentiated) cells[6,7]. Ganglioside GM3 promotes cell proliferation, migration and tumorigenesis while promoting cancer cell resistance to anti-cancer drug therapy[8-10]. Depending on the concentration, ganglioside GD3 exhibits a diversity of effects, such as inhibiting cell growth, inducing apoptosis, enhancing radiation and anti-cancer drug therapy efficacy and exerting anti-inflammatory effects[11]. Change in ganglioside composition relies on the balance between activities of enzymes in ganglioside biosynthetic and degradative pathways [6,12,13]. A simplified diagram of ganglioside biosynthesis and degradation is illustrated (Figure 3-1).

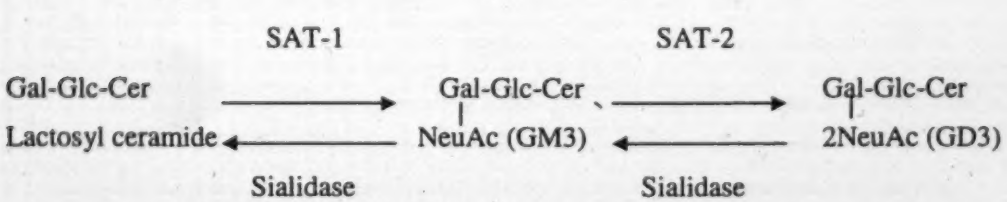


Figure 3-1 Simplified Schematic of Ganglioside Biosynthesis and Degradation
Abbreviations: Gal = galactose; Glc = glucose; Cer = ceramide; NeuAc = n-acetyl neuraminic acid or sialic acid; SAT-1 = sialyltransferase 1 or GM3 synthase; SAT-2 = sialyltransferase 2 or GD3 synthase

Sialyltransferase 1 (SAT-1) drives lactosylceramide towards GM3 synthesis while sialyltransferase 2 (SAT-2) catalyses the biosynthesis of GD3 (Figure 3-1). GM3 and GD3 are desialylated by a group of enzymes called the sialidases which are classified based on their location in the cell (1 = lysosome, 2 = cytosol, 3 = plasma membrane and 4 = mitochondria)[14]. In a recent study, the expression of SAT-1 and SAT-2 mRNA were found to be lower in tumor tissues from patients with colorectal cancer than in corresponding healthy tissues[15]. In contrast, the expression of human plasma membrane sialidase, an enzyme involved in removing terminal sialic acid from gangliosides, was found to be up-regulated in colon cancer tissue and fetal colon[12]. Furthermore, differentiation of colon cancer cell lines (metastatic to poorly metastatic phenotype) and susceptibility to apoptosis was found to be associated with a decrease in human plasma membrane sialidase expression and activity[12]. Thus, it would be expected that a differentiating colon cancer cell would have low amounts of mitotic GM3 and high amounts of apoptotic GD3 and complex gangliosides, a ganglioside profile similar to that of human colostrum and neonatal rat intestine[13,16,17]. Despite evidence for similar changes in expression of enzymes involved in ganglioside synthesis and degradation during development, lactation and oncogenic transformation, it has not been studied whether ganglioside compositional changes in differentiating colon cancer cells reflect changes that occur during intestine development.

CaCo-2 cells are a human colon cancer cell line isolated from a 72 year-old Caucasian male presenting with an adenocarcinoma of the colon[18]. In a study of 20 human colon tumor cell lines, CaCo-2 alone showed the ability to undergo spontaneous differentiation to develop a number of characteristics more commonly associated with small intestinal enterocytes[19]. The development of the enterocyte-like phenotype is only evident when the cells reach confluence. During the 7 to 20 day post confluence differentiation time the cell monolayer gradually develops brush border microvilli, tight junctions, cell polarity and expression of typical small intestinal microvillus hydrolases and nutrient transporters[20-22]. Moreover, *Bifidobacteria* and *Lactobacilli* species are able to adhere to CaCo-2 cells and competitively exclude enterotoxigenic *E. coli* and *S. typhimurium* indicating that CaCo-2 cells are a beneficial cell line for studying bacteria colonization of the gut[23,24]. CaCo-2 cells exhibit differences in polarity and expression

of proteins depending on the time frame for differentiation, suggesting the potential use of CaCo-2 cells as a model for different stages of intestinal development. Obtaining sufficient amounts of infant intestine is ethically difficult and isolated primary cultures of enterocytes have a limited survival time in culture, necessitating an alternative model for neonatal intestine.

The present study was designed to determine whether differentiating CaCo-2 cells acquire a ganglioside composition profile similar to human colostrum and neonatal rat intestine, thereby resulting in a decrease in the GM3 to GD3 ratio. The ganglioside composition of undifferentiated and differentiated CaCo-2 cells has not been assessed. Considering that the CaCo-2 cell line is the most widely used cell line for studying physiological and pathophysiological processes in the small intestine and colon, understanding the change in ganglioside composition during differentiation is important to assess the potential use of differentiating CaCo-2 cells as a model for studying intestine development and pediatric intestinal disorders.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture

Human colon cancer CaCo-2 cells (passage 44-54) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Earle's Minimum Essential Medium (EMEM) containing 10% (v/v) fetal bovine serum (FBS), 1% (v/v) antibiotic/antimycotic, 26 mM sodium bicarbonate, 10 mM HEPES and 1 mM pyruvic acid. Cells were grown as adherent monolayers in 75 cm^2 T-flasks under standard incubator conditions (humidified atmosphere, 5% CO_2, 37°C) with medium replaced every 2-3 days. Monolayers were subcultured on reaching 80-90% confluence at a split ratio of 1:3 (1 T75 flask and 2 T150 flasks) using 0.25% trypsin/0.03% ethylenediaminetetraacetic acid (EDTA).

For each ganglioside composition experiment, 16 confluent T150 flasks of undifferentiated cells and four 20 day post confluent T150 flasks of differentiated cells were collected in cold Tris buffer/EDTA wash using a cell scrapper and were pooled together into an undifferentiated group and a differentiated group of CaCo-2 cells. Cell suspensions were centrifuged for 10 min at 1000 rpm and the resulting cell pellet was

lysed in 0.5 ml of 2mM Tris HCl/40 mM mannitol lysis buffer and sonicated for 30 s on ice. Cell homogenates were centrifuged for 10 min at 12,000 rpm and cell supernatants were saved for protein and ganglioside analysis.

3.2.2 Determination of cell protein

The amount of protein in cell supernatants was determined using the bicinchoninic acid (BCA) assay. Cell supernatants were diluted 1 in 5 with ddH_2O. Bovine serum albumin standards and diluted cell supernatants (10 µl) were each mixed with 190 µl of a 50:1 mixture of BCA solution and 4% (w/v) $CuSO_4 \cdot 5H_2O$ for 30 min at 37°C. The absorbance at 562 nm was measured with a microplate reader (Molecular Devices Co., USA).

3.2.3 Assessment of cell differentiation markers

CaCo-2 cells were seeded on inserts in 12 well trans-well plates at a density of 400 000 cells per well in 0.5 ml of EMEM-10. The bottom compartment received 1.5 ml of EMEM-10. Trans-epithelial resistance was measured prior to media change every 2-3 days for 30 days with a voltmeter to access monolayer polarity as a marker for cell differentiation.

Alkaline phosphatase activity of undifferentiated and differentiated CaCo-2 cells was measured as a marker of cell differentiation. For each alkaline phosphatase activity experiment, 1 T150 flask each of undifferentiated (confluence) and differentiated (10 day and 20 day post confluence) CaCo-2 cells was collected and cell homogenates were prepared in Tris mannitol buffer with sonication as described previously in the cell culture methods section. Cell homogenates and p-nitrophenol standards (10 µl) were added to wells in a 96 well plate and mixed with 190 µl of alkaline phosphatase reagent for 30 min at 37°C. The reaction was stopped with 2N NaOH and the absorbance at 405 nm was measured with a microplate reader.

3.2.4 Ganglioside extraction

Total lipid was extracted from cell supernatants using the Folch method[25]. In short, 0.9 ml aliquots of cell supernatant were mixed with 18 ml of

chloroform/methanol (2:1, v/v) and incubated overnight on a shaker. Distilled water was added to give a final ratio of 5:1 chloroform methanol (2:1, v/v)/water. The upper aqueous phase containing gangliosides was collected. To increase the yield of gangliosides, the lower organic phase was washed twice with Folch upper phase solution (chloroform/methanol/water, 3:48:47 by vol.). The upper aqueous phases containing gangliosides were pooled together and purified by passage through Sep-Pak C_{18} cartridges (Waters Corporation, Milford, MA, USA) pre-washed with 10 ml methanol, 20 ml chloroform/methanol (2:1, v/v) and 10 ml of methanol as described by Williams and McCluer[26]. The upper phase extract was loaded onto C_{18} cartridges. Cartridges were washed with 20 ml of distilled water to remove salts and water-soluble contaminants. Gangliosides were eluted with 5ml of methanol and 20 ml of chloroform/methanol (2:1, v/v), dried under nitrogen gas and redissolved in 500 µl of chloroform/methanol (2:1, v/v). Gangliosides were stored at -20°C until analysis.

3.2.5 Analysis of total and individual ganglioside content

Total gangliosides were measured as ganglioside-bound N-acetyl neuraminic acid (GG-NANA) as described by Suzuki[27]. A 100 µl aliquot of purified ganglioside sample was dried under nitrogen gas and dissolved with 0.5 ml of distilled water and 0.5 ml of resorcinol-HCl in screw-capped Teflon-lined tubes. The purple blue color developed by heating the sample for 8 min at 150-160°C was extracted into butylacetate/butanol (85:15, v/v) solvent. Optical density was read by a spectrophotometer (Hewlett Packard, 8452A) at 580 nm. For quantitative analysis, N-acetyl neuraminic acid (Sigma, MO, USA) was used as a standard.

The remaining 400 µl of sample ganglioside was dried under nitrogen and redissolved in 100 µl of chloroform/methanol (2:1, v/v). Individual gangliosides were separated by silica gel high performance thin layer chromatography (HPTLC; Whatman Inc, Clifton, NJ, USA) along with ganglioside standards GM3, GD3 and bovine brain ganglioside mixture (Alexis, San Diego, CA, USA) in a solvent system of chloroform/methanol/0.2% (w/v) $CaCl_2 \cdot 2H_2O$ (55:45:10, by vol.). Individual gangliosides were visualized under ultraviolet light by spraying HPTLC plates with 0.1% (w/v) 8-anilino-1-naphthalene-sulfonic acid (ANSA). Each ganglioside band was

scraped into a glass tube. Gangliosides were eluted from silica by vortex, sonication and shaking overnight in 10 ml of chloroform/methanol (2:1, v/v). Tubes were centrifuged for 10 min at 1000 rpm to spin down the silica. The chloroform/methanol (2:1, v/v) phase was collected and combined with a 10 ml chloroform/methanol (2:1, v/v) wash and a 5 ml methanol wash of the silica. Individual gangliosides were measured as ganglioside bound N-acetyl neuraminic acid as described above. To determine the percentage of individual gangliosides in the mixture, gangliosides separated on HPTLC plates were sprayed with resorcinol-HCl and heated for 7-10 min at 150-160°C to visualize purple ganglioside bands. Each ganglioside band was quantified as a percentage of the total gangliosides by densitometry (Beckman CDS-200; Beckman Coulter, Mississauga, ON, Canada) using Quantity One software (Biorad Laboratories Inc, Hercules, CA, USA).

3.2.6 Statistical analysis

Sample size determination for a one-tailed comparison at 80% power to detect a 2 fold increase in liver gangliosides (44.3 nmol/g wet weight to 79.1 nmol/g wet weight, $p<0.01$) requires a sample size of 3[28]. All values are displayed as the mean ± the standard error of the mean for a sample size of n = 6 (6 different passages of cells) for individual gangliosides measured by the colorimetric, N-acetyl neuraminic acid assay, an n=5 (5 different passages of cells) for total gangliosides and an n = 4 (4 different passages of cells) for individual gangliosides measured by densitometry and differentiation markers. Significant differences in amount and composition of gangliosides between undifferentiated and differentiated CaCo-2 cells was determined by a one-way analysis of variance (ANOVA) and a Tukey test with SAS statistical software (SAS Institute Inc, Version 9.1, NC, USA). A *P* value of less than 0.05 was considered statistically significant.

3.3 RESULTS

3.3.1 Trans-epithelial resistance and alkaline phosphatase activity of undifferentiated and differentiated CaCo-2 cells

Trans-epithelial resistance was measured when CaCo-2 cells reached confluence and every 3 days post confluence up to 30 days to monitor cell polarity as a marker of cell differentiation. As indicated in Figure 3-2A, trans-epithelial resistance displayed a linear increase over time as CaCo-2 cells differentiated. Alkaline phosphatase activity was measured when CaCo-2 cells reached confluence and 10 and 20 days post confluence as a second marker of cell differentiation. As indicated in Figure 3-2B, alkaline phosphatase activity was significantly higher 10 and 20 days post confluence compared to undifferentiated, confluent CaCo-2 cells.

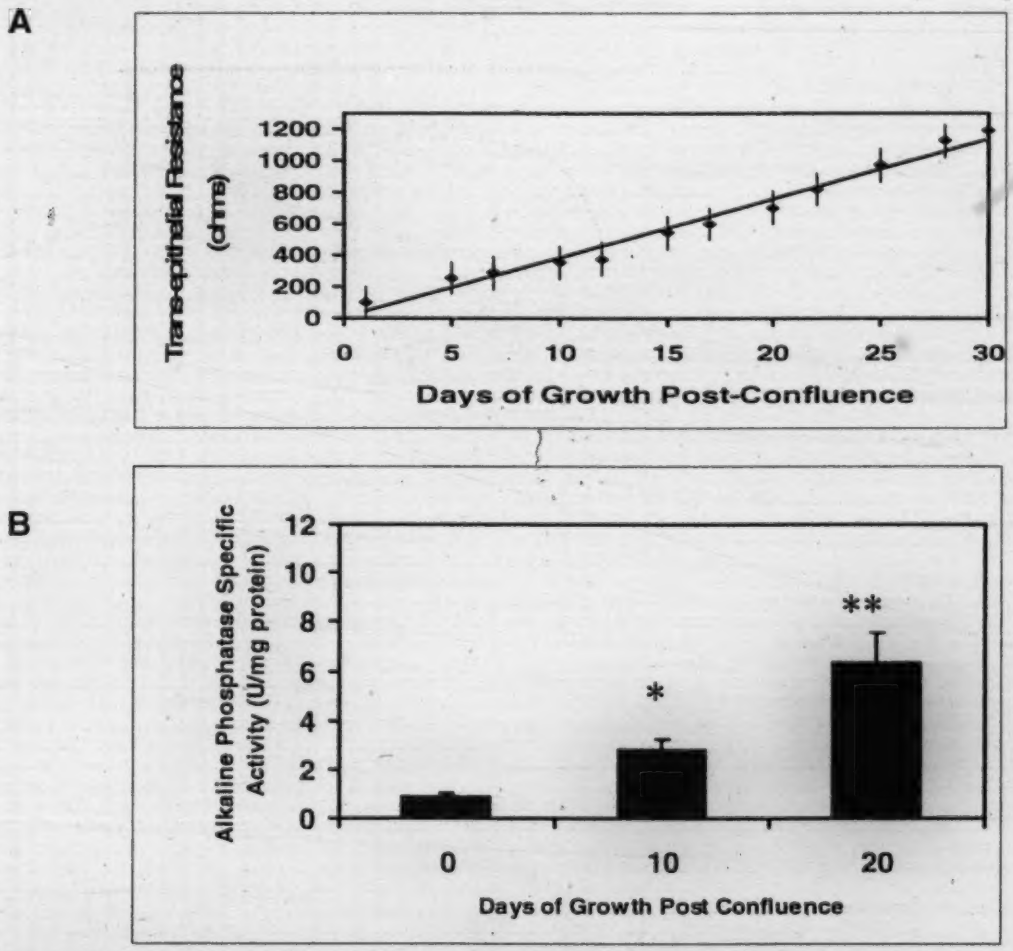


Figure 3-2 Differentiation markers **A** trans-epithelial resistance and **B** alkaline phosphatase specific activity for CaCo-2 cells measured at confluence (undifferentiated cells) and post confluence (differentiated cells). Values represent the mean ± the standard error of the mean for a sample size of n=4 (4 different cell passages). * $P<$ 0.001 and ** $P \leq 0.0001$.

3.3.2 Total ganglioside content of undifferentiated and differentiated CaCo-2 cells

The influence of differentiation on total ganglioside content of human colon cancer CaCo-2 cells is shown in Figure 3-2. CaCo-2 cells differentiated for 20 days had 2.5 times higher ganglioside content compared to undifferentiated CaCo-2 cells.

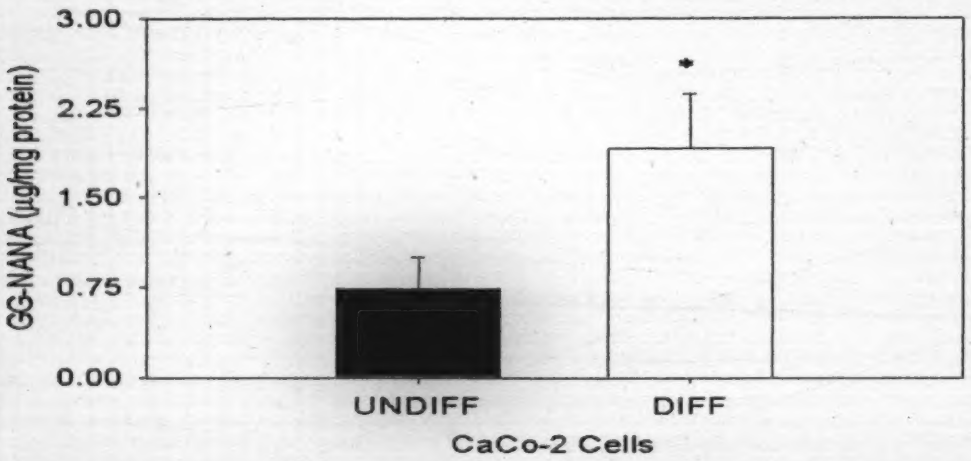
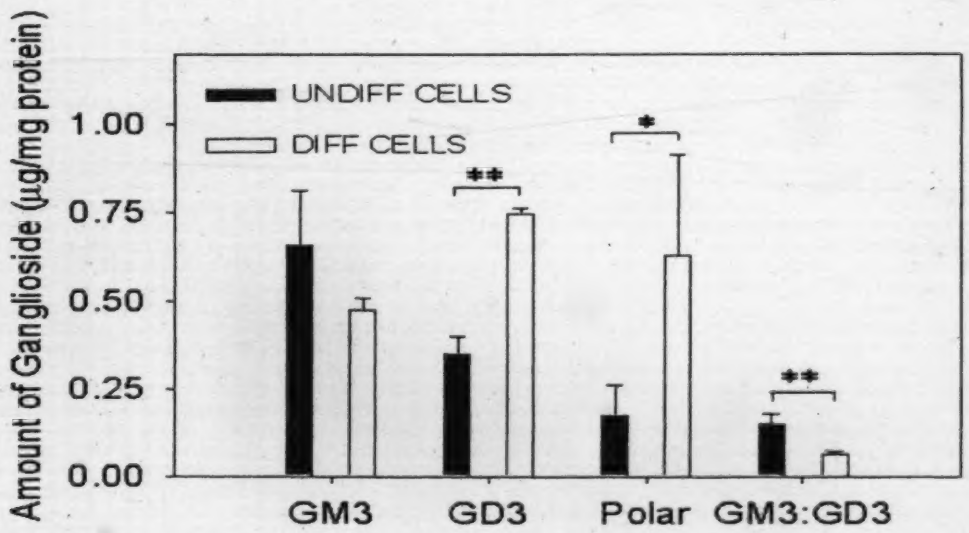


Figure 3-3 Total content of ganglioside-bound N-acetyl neuraminic acid (GG-NANA) in undifferentiated (UNDIFF) and differentiated (DIFF) human colon cancer CaCo-2 cells grown to confluence or differentiated 20 days post confluence, respectively. Values are means ± the standard error of the mean for a sample size of n=5 (5 different cell passages). * $P < 0.05$.

3.3.3 Quantification of individual ganglioside composition in undifferentiated and differentiated human colon cancer CaCo-2 cells

The amount and percent of individual gangliosides as well as changes in the GM3:GD3 ratio of undifferentiated and differentiated CaCo-2 cells measured using a colorimetric N-acetyl neuraminic acid (NANA) assay and scanning densitometry are illustrated (Figure 3-4). Differentiated CaCo-2 cells had a significantly higher amount of GD3 and polar gangliosides and a trend towards lower amounts of GM3 compared to undifferentiated cells (Figure 3-4a). Moreover, the percentage GD3 and polar gangliosides was also significantly higher in differentiated cells (Figure 3-4b). Undifferentiated cells had a higher percentage of GM3 and GM2 (Figure 3-4b). Independent of the method used to quantify individual gangliosides, the ratio of GM3:GD3 decreased when CaCo-2 cells were differentiated and the drop in the ratio was significant when ganglioside was quantified using the NANA assay (Fig 3-4a, b).

A



B

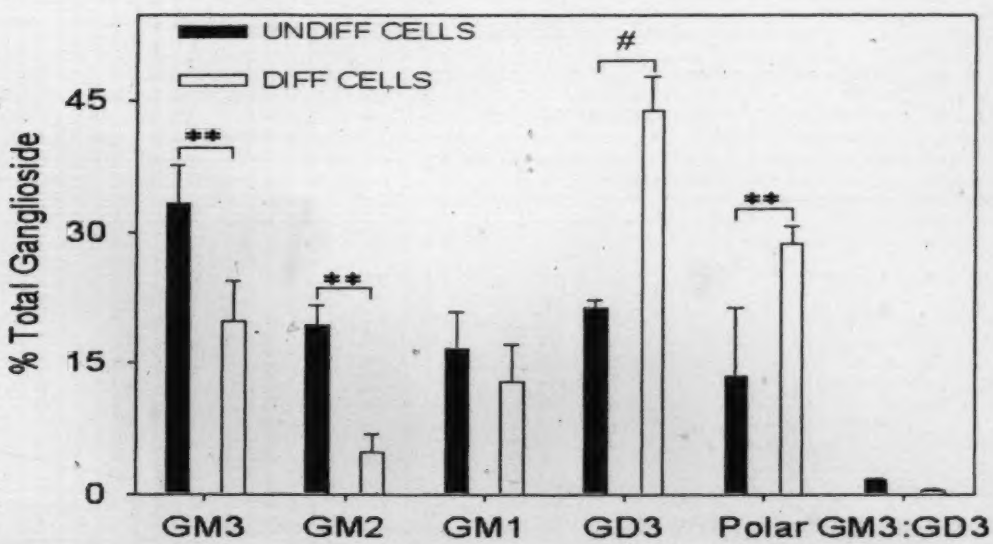


Figure 3-4 Ganglioside composition of undifferentiated and differentiated CaCo-2 cells measured by a colorimetric N-acetyl neuraminic acid (NANA) assay (A) and scanning densitometry (B). Values are expressed as the mean ± the standard error of the mean for six different cell passages (n = 6) for (A) and n = 4 for (B). * $P < 0.01$; ** $P \leq 0.005$; # $P \leq 0.0001$.

3.4 DISCUSSION

The discovery that changes in glycosphingolipid metabolism are similar during development and oncogenic transformation prompted research on the characterization of ganglioside content and composition during different stages of cell differentiation. Differentiating nerve and leukemia cell lines have been investigated thoroughly; however, knowledge on the ganglioside content and composition of differentiating intestinal cells is limited[8,29]. With increasing evidence that parallel changes in ganglioside content and composition occur during lactation and intestinal development[13,17,30], it became of interest to investigate whether ganglioside alterations during intestinal cancer cell differentiation resemble the compositional changes during lactation and intestinal development. A comparable shift in ganglioside content and composition would demonstrate the potential use of differentiating CaCo-2 cells as a model for studying the physiology and pathophysiology of neonatal gut.

The present study investigated the ganglioside content and composition of undifferentiated and differentiated human colon cancer CaCo-2 cells. This study demonstrated that differentiated human colon cancer CaCo-2 cells have 2.5 times more total gangliosides than poorly differentiated CaCo-2 cells. The trend for enhanced ganglioside content following differentiation is supported by the observation that ganglioside sugar chain elongation is enhanced in differentiated human colon cancer HT-29 cells[31]. Moreover, lipid bound sialic acid levels are significantly elevated in hepatoma tissues during proliferation and differentiation[32]. Similar to differentiated colon cancer cells, human colostrum has twice as many gangliosides as mature milk and a larger fraction of complex gangliosides with branched sugar chains[13]. Although the ganglioside content of infant bowel has not been investigated, it is known that neonatal rat intestine contains more gangliosides than adult rat intestine and that the ganglioside composition varies along the crypt-villus axis[16,33]. Accumulation of individual gangliosides in colostrum, immature intestine and differentiated intestinal cells suggests a unique physiological role for specific gangliosides in cell development and differentiation.

Individual ganglioside composition of undifferentiated and differentiated CaCo-2 cells was determined and compared to human colostrum and immature rat intestine to

elucidate possible functions for specific gangliosides in development and differentiation. In the present study, ganglioside accumulation in differentiated CaCo-2 cells was accompanied by changes in the individual ganglioside composition. Differentiated CaCo-2 cells contained more GD3 and polar, complex gangliosides than undifferentiated CaCo-2 cells. Human colostrum and differentiated human embryonic stem cells also contain high amounts of GD3 and polar, complex gangliosides[17,34]. The accumulation of GD3 and polar gangliosides in colostrum, differentiated stem cells and differentiated colon cancer cells may support a role for GD3 and polar gangliosides in promoting cell proliferation, growth, intestine development and enterocyte differentiation. The effect of gangliosides on cell proliferation and motility are differential and depend on cell type and ganglioside associated molecules in the individual cell types[35]. In CaCo-2 cells, calcium sensing receptors that regulate cell proliferation and are modulated by gangliosides are most concentrated in crypt cells and differentiated colon cancer lesions[36]. Accumulation of GD3 and polar gangliosides in cells with receptors that regulate cell proliferation and growth suggests a role for GD3 and polar gangliosides in promoting cell proliferation during development and tumor progression.

Differentiated CaCo-2 cells also contained a lower percentage of GM3 when compared to undifferentiated CaCo-2 cells. This ganglioside compositional change was accompanied by a small decrease in the GM3:GD3 ratio. Nojiri demonstrated that differentiated HCT-116 colon cancer cells lose tumorigenic activity and become susceptible to apoptosis by artificially increasing GM3 content[37]. Differentiated leukemia cells and macrophages have been shown to have elevated levels of GM3[38,39]. Unlike some other differentiated cell lines, the differentiated CaCo-2 cell did not have a significantly different GM3 content than the undifferentiated form. The differentiated CaCo-2 cell also had a lower percentage of GM3 and GM2. Thus, the decrease in the GM3:GD3 ratio is attributed to an increase in GD3 content with a small decline in GM3. Perhaps differentiating CaCo-2 cells up-regulate sialyltransferase 2 (SAT-2) and convert GM3 into GD3 with hydrolysis of GM2 replenishing some of the lost GM3. Several studies have observed an elevation in the GD3 synthesis level of apoptotic cells[40-42]. Saha demonstrated that the level of GM2 and GM3 correlates with metastatic

potential[43]. Furthermore, differentiation of colon cancer cell lines (metastatic to poorly metastatic phenotype) and susceptibility to apoptosis was found to be associated with a decrease in human plasma membrane sialidase expression and activity[12]. Taken together with previous studies, the susceptibility of differentiated CaCo-2 cells to apoptosis may be explained by elevated apoptotic GD3 while malignancy may be promoted by suppressing apoptosis through hydrolysis of GD3.

The difference in the GM3:GD3 ratio between undifferentiated and differentiated CaCo-2 cells was small in comparison to the large changes reported in the literature during lactation. Depending on the ganglioside analysis method, undifferentiated CaCo-2 cells had a GM3:GD3 ratio that was 2.4-3.5 times higher than the GM3:GD3 ratio of differentiated CaCo-2 cells. In a study that followed the change in ganglioside composition of human milk over time, the GM3:GD3 ratio was reported to be 70 times greater in mature milk when compared to colostrum (0.05 colostrum vs. 3.5 mature milk)[44]. The shift in the ratio during lactation was associated with a large increase in GM3 levels along with a large decrease in GD3 levels[13]. The GM3:GD3 ratio of mature rat small intestine is 26, similar to the ratio of 19.8 observed in mature milk[13,45]. For neonatal rat small intestine, the GM3:GD3 ratio has not been determined; however it is known that neonatal tissues decrease GD3 content and increase GM3 content during development[16,17,34]. Although the ganglioside composition changes are similar during rat intestine development, lactation and oncogenic transformation of colon cells, further ganglioside analysis work should be completed with infant bowel to determine changes in the GM3:GD3 ratio during development and look at the degree of change in GM3 and GD3 levels. The changes in ganglioside content and composition of CaCo-2 cells should also be measured in partially differentiated CaCo-2 cells and confirmed with a more sensitive ganglioside analysis method such as liquid chromatography/tandem mass spectrometry to more accurately access individual ganglioside composition[46]. Adapting a more sensitive ganglioside analysis method would decrease tissue requirements, eliminate cell pooling and help clarify the changes that occur in individual species of polar, complex gangliosides that are often present in small amounts.

13. Takamizawa, K., Iwamori,.M., Mutai, M. & Nagai, Y. Selective changes in gangliosides of human milk during lactation: a molecular indicator for the period of lactation. *Biochim Biophys Acta* 879, 73-7 (1986).
14. Yamanami, H. et al. Down-regulation of sialidase NEU4 may contribute to invasive properties of human colon cancers. *Cancer Sci* 98, 299-307 (2007).
15. Gornati, R. et al. Evaluation of SAT-1, SAT-2 and GalNAcT-1 mRNA in colon cancer by real-time PCR. *Mol Cell Biochem* (2006).
16. Bouhours, J.F., Bouhours, D. & Hansson, G.C. Developmental changes of glycosphingolipid composition of epithelia of rat digestive tract. *Adv Lipid Res* 26, 353-72 (1993).
17. Rueda, R., Maldonado, J., Narbona, E. & Gil, A. Neonatal dietary gangliosides. *Early Hum Dev* 53 Suppl, S135-47 (1998).
18. Fogh, J., Fogh, J.M. & Orfeo, T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* 59, 221-6 (1977).
19. Chantret, I., Barbat, A., Dussaulx, E., Brattain, M.G. & Zweibaum, A. Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of twenty cell lines. *Cancer Res* 48, 1936-42 (1988).
20. Chantret, I. et al. Differential expression of sucrase-isomaltase in clones isolated from early and late passages of the cell line Caco-2: evidence for glucose-dependent negative regulation. *J Cell Sci* 107 (Pt 1), 213-25 (1994).
21. Harris, D.S., Slot, J.W., Geuze, H.J. & James, D.E. Polarized distribution of glucose transporter isoforms in Caco-2 cells. *Proc Natl Acad Sci U S A* 89, 7556-60 (1992).
22. Peterson, M.D., Bement, W.M. & Mooseker, M.S. An in vitro model for the analysis of intestinal brush border assembly. II. Changes in expression and localization of brush border proteins during cell contact-induced brush border assembly in Caco-2BBe cells. *J Cell Sci* 105 (Pt 2), 461-72 (1993).
23. Bernet, M.F., Brassart, D., Neeser, J.R. & Servin, A.L. Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. *Appl Environ Microbiol* 59, 4121-8 (1993).
24. Duffy, L.C. Interactions mediating bacterial translocation in the immature intestine. *J Nutr* 130, 432S-436S (2000).
25. Folch, J., Lees, M. & Sloane Stanley, G.H. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226, 497-509 (1957).
26. Williams, M.A. & McCluer, R.H. The use of Sep-Pak C18 cartridges during the isolation of gangliosides. *J Neurochem* 35, 266-9 (1980).
27. Suzuki, Y., Suzuki, N., Michi, H. & Matsumoto, M. Characterization of gangliosides of porcine erythrocyte membranes: occurrence of ganglioside GD3 as major ganglioside. *Lipids* 20, 588-93 (1985).
28. Majer, F. et al. Estrogen-induced cholestasis results in a dramatic increase of b-series gangliosides in the rat liver. *Biomed Chromatogr* 21, 446-50 (2007).
29. Varner, J.A. Stem cells and neurogenesis in tumors. *Prog Exp Tumor Res* 39, 122-9 (2007).

30. Nakano, T., Sugawara, M. & Kawakami, H. Sialic acid in human milk: composition and functions. *Acta Paediatr Taiwan* 42, 11-7 (2001).
31. Houri, J.J., Falbo, A., Vignali, G., Codogno, P. & Ghidoni, R. The metabolic processing of glycosphingolipids in HT-29 cells is differentiation-dependent. *Biochem Biophys Res Commun* 202, 992-1000 (1994).
32. Lu, C.Q., Lu, J., Wang, B.L. & Cui, Y.Z. Changes in ganglioside contents, plasma sialic acid and cAMP levels in experimental hepatoma in mice. *Mol Cell Biochem* 207, 29-33 (2000).
33. Glickman, R.M. & Bouhours, J.F. Characterization, distribution and biosynthesis of the major ganglioside of rat intestinal mucosa. *Biochim Biophys Acta* 424, 17-25 (1976).
34. Kwak, D.H. et al. Dynamic changes of gangliosides expression during the differentiation of embryonic and mesenchymal stem cells into neural cells. *Exp Mol Med* 38, 668-76 (2006).
35. Kamimura, Y. et al. Differential enhancing effects of alpha2,8-sialyltransferase on the cell proliferation and mobility. *Int J Oncol* 26, 337-44 (2005).
36. Kallay, E. et al. Dietary calcium and growth modulation of human colon cancer cells: role of the extracellular calcium-sensing receptor. *Cancer Detect Prev* 24, 127-36 (2000).
37. Nojiri, H., Yamana, H., Shirouzu, G., Suzuki, T. & Isono, H. Glycotherapy for cancer: remodeling of ganglioside pattern as an effective approach for cancer therapy. *Cancer Detect Prev* 26, 114-20 (2002).
38. Chung, T.W., Choi, H.J., Lee, Y.C. & Kim, C.H. Molecular mechanism for transcriptional activation of ganglioside GM3 synthase and its function in differentiation of HL-60 cells. *Glycobiology* 15, 233-44 (2005).
39. Nojiri, H., Takaku, F., Terui, Y., Miura, Y. & Saito, M. Ganglioside GM3: an acidic membrane component that increases during macrophage-like cell differentiation can induce monocytic differentiation of human myeloid and monocytoid leukemic cell lines HL-60 and U937. *Proc Natl Acad Sci U S A* 83, 782-6 (1986).
40. Bennaceur, K., Popa, I., Portoukalian, J., Berthier-Vergnes, O. & Peguet-Navarro, J. Melanoma-derived gangliosides impair migratory and antigen-presenting function of human epidermal Langerhans cells and induce their apoptosis. *Int Immunol* 18, 879-86 (2006).
41. Hasegawa, T. et al. Role of Neu4L sialidase and its substrate ganglioside GD3 in neuronal apoptosis induced by catechol metabolites. *FEBS Lett* 581, 406-12 (2007).
42. Omran, O.M., Saqr, H.E. & Yates, A.J. Molecular mechanisms of GD3-induced apoptosis in U-1242 MG glioma cells. *Neurochem Res* 31, 1171-80 (2006).
43. Saha, S. & Mohanty, K.C. Correlation of gangliosides GM2 and GM3 with metastatic potential to lungs of mouse B16 melanoma. *J Exp Clin Cancer Res* 22, 125-34 (2003).
44. Pan, X.L. & Izumi, T. Chronological changes in the ganglioside composition of human milk during lactation. *Early Hum Dev* 55, 1-8 (1999).
45. Park, E.J. et al. Diet-induced changes in membrane gangliosides in rat intestinal mucosa, plasma and brain. *J Pediatr Gastroenterol Nutr* 40, 487-95 (2005).

46. Sorensen, L.K. A liquid chromatography/tandem mass spectrometric approach for the determination of gangliosides GD3 and GM3 in bovine milk and infant formulae. *Rapid Commun Mass Spectrom* 20, 3625-33 (2006).

CHAPTER 4

GANGLIOSIDE GD3 ABSORPTION BY HUMAN INTESTINE CACO-2 CELLS

4.1 INTRODUCTION

The intestinal barrier is comprised of specialized epithelial cells that form tight junctions and selectively allow movement of nutrients from the intestinal lumen into the circulation while impeding penetration of harmful entities[1]. The ability of nutrients to enter the enterocyte and cross the intestinal barrier is an important consideration when designing therapeutic agents for gastrointestinal disorders or for intravenous delivery to other tissues. In vitro intestinal barrier model systems such as the enterocyte-like human colon cancer CaCo-2 cell line provide an efficient method to screen compounds for their ability to cross the intestinal barrier[2,3]. CaCo-2 cells spontaneously differentiate into small intestinal-like enterocyte monolayers upon reaching confluence[4,5] and following a 20 day post confluent culture time develop brush border microvilli, tight junctions, cell polarity and expression of typical small intestinal microvillus hydrolases and nutrient transporters[6-8]. Culturing cells on porous membranes permits independent manipulation of the local environment at either the apical or basolateral pole of the cell and allows for measure of transfer of nutrients across membranes providing a more complete assessment of nutrient bioavailability. CaCo-2 cells grown on trans-well inserts are a well established model and have been used in many applications including drug and nutrient screening for bioavailability and investigation of toxin, immune cell or microbe translocation across the intestine including mechanism of action[9-12]. Nevertheless, it is important to consider that CaCo-2 cells are immortalized having been derived from an adenocarcinoma patient and their ability to shed gangliosides must be accounted for[13]. Unfortunately, primary cultures of enterocytes derived from healthy intestine tissue exhibit limited survival time in culture and ethically it is difficult to obtain significant amounts of human intestine tissue for research. Thus, the human CaCo-2 cell line remains the model of choice for studying nutrient bioavailability.

Gangliosides are sialic acid-containing glycosphingolipids concentrated predominantly in lipid rafts of the outer plasma membrane where the oligosaccharide portion is exposed toward the cell surface and the hydrophobic ceramide is inserted into

the surface layer of the membrane[14]. In rat intestine, gangliosides make up 34% of glycosphingolipids with the predominant ganglioside of mature intestine, GM3, localized in the brush border membrane (BBM) and the predominant ganglioside found in neonatal intestine, GD3, found in the basolateral membrane (BLM)[15,16]. The concentration of gangliosides is lowest in the proximal intestine and increases along the length of rat intestine toward the distal segment[17]. Gangliosides are also found in subcellular organelles of glycosphingolipid metabolism (endosomes, ER, Golgi apparatus, lysosomes), plasma lipoproteins and the milk fat globule membrane[14,18,19]. Although present in small amounts in the diet[20], dietary gangliosides are incorporated into tissues and exert potent effects on cellular functions[16,21]. Depending on location, concentration and form, individual gangliosides play important roles in recognition, attachment and translocation of cells, microbes and macromolecules across membranes and regulate cell signaling and protein function[19,22-26]. For example, ganglioside GD3 is particularly abundant in colostrum, developing tissues and several tumors[19,27,28]. GD3 suppresses inflammation and is involved in cell proliferation and differentiation during development[19,21]. The role of GD3 in tumor growth and metastasis is controversial. GD3 induces apoptosis and sensitizes tumor cells to anti-cancer drug therapy[29,30] while GD3 also promotes angiogenesis and suppresses immune system tumor killing[31]. In addition to GD3 concentration, acetylation is a determinant of GD3 function[32,33]. As protective mechanisms, cells tightly regulate ganglioside levels in the body by intracellular compartmentalization, rapid turnover and chemical modification[34].

To date there are no studies that have specifically looked at the mechanism or efficiency of ganglioside uptake and transfer into and across the intestine. It is known that gangliosides form aggregates in aqueous environments such as the unstirred water layer and depending on their composition form stable micelles or unilamellar vesicles[35,36]. Cell culture studies suggest that exogenously administered gangliosides are taken up by a wide range of cells via two different mechanisms: 1) molecules dissociated from micelles, diffuse through the aqueous phase and insert into plasma membranes with or without a protein carrier 2) micelles are taken up by receptor-mediated endocytosis[36]. According to Pagano's vesicle sorting theory[37], absorbed gangliosides have three fates: transport back to the plasma membrane immediately after

being endocytosed; endocytosis to the Golgi apparatus for glycosylation to form more complex ganglioside species and transport by the endosome to the lysosome for degradation into bioactive mediators such as sphingosine and ceramide. Recently, glycolipid transport proteins have been identified in the cytosol that may play a role in non-vesicular transport of glycolipids within cells[38]. Considering that rats fed a diet enriched in ganglioside GD3 have elevated levels of GD3 in intestinal mucosa lipid rafts and plasma[16], it is reasonable to assume that gangliosides are absorbed by the intestine; that is GD3 is both taken up across the brush border membrane into the enterocyte and is transferred across the basolateral membrane into the blood. The capability of tumor cells to shed gangliosides as micelles, monomers or membrane vesicles suggests a mechanism exists for removal of cell surface gangliosides[13]. A schematic summarizing proposed mechanisms of ganglioside absorption by the intestine and intracellular fate of absorbed ganglioside is shown (Figure 4-0).

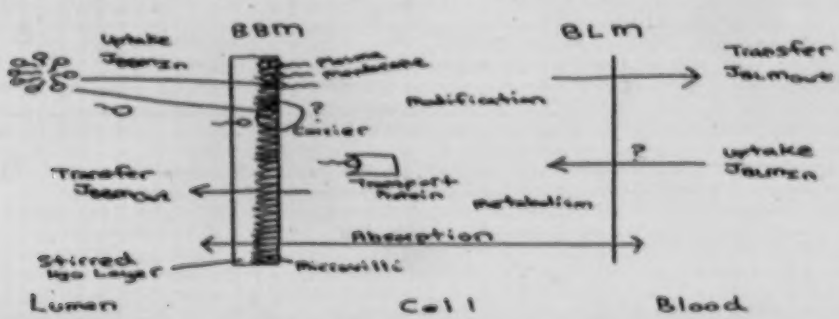


Figure 4-0 Mechanisms proposed for ganglioside absorption by intestine and intracellular fate of absorbed gangliosides. Micellar gangliosides are taken up from the lumen either by receptor mediated endocytosis or dissociation of gangliosides from micelles which diffuse across the unstirred water layer and insert into the brush border membrane (BBM). The uptake process may or may not require a carrier. Once inserted within the intestinal cell membrane, gangliosides are mobilized through a series of metabolic pathways. Some intact gangliosides stay in the outer plasma membrane and some are transported within the cell by endosomes or glycolipid transport proteins. Gangliosides may be targeted to lysosomes for metabolism and release of bioactive by-products. Alternatively, gangliosides may be chemically modified (i.e. glycosylated, sialylated, acetylated) in the Golgi apparatus or transferred across the basolateral membrane (BLM) into the blood for transport to peripheral tissues. Gangliosides may be transported bound to albumin or within plasma lipoproteins. Uptake of gangliosides from the blood has not been studied. Shedding of surface gangliosides by tumor cells suggests a possible mechanism for transferring gangliosides across the BBM into the intestinal lumen. Absorption is the sum of ganglioside uptake and transfer from one side of the intestine to the other. Abbreviation: J = flux.

It is not known whether ganglioside uptake and transfer involves a carrier, whether the processes are efficient and what the fate is for absorbed ganglioside. Furthermore, the uptake and transfer of gangliosides delivered on the BLM side of intestine has not been investigated. It is essential to understand ganglioside absorption mechanism and fate in order to efficiently deliver enteral or intravenous gangliosides to the gut to enhance development or treat intestinal diseases.

Thus, it is highly relevant to study the absorption of ganglioside GD3 by human intestine CaCo-2 cells. In this study, we determined the GD3 concentration range that does not exhibit toxicity to enterocytes and compared the time frame, concentration effect, mechanism and efficiency of GD3 absorption from both the apical and basolateral membrane side of polarized human intestine CaCo-2 cell monolayers. Moreover, the fate of absorbed ganglioside was investigated by determining the proportion of GD3 retained, metabolized or transferred across the enterocyte following GD3 uptake. It was hypothesized that enterocyte uptake of GD3 would be efficient from both the apical and basolateral side and that route of delivery would influence GD3 uptake efficiency and fate. We demonstrate that GD3 uptake into enterocytes is both time- and concentration-dependent and reaches a plateau. Finally, this study shows that fate of ganglioside taken up by the intestine depends on the route in which it was delivered. Basolateral GD3 is destined for metabolism by the enterocyte while apical GD3 is metabolized with some cellular retention in the membrane and some transfer across the basolateral membrane for distribution to other tissues.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Materials

Zeta dairy lipid powder was supplied by Fonterra (Cambridge, New Zealand). All cell culture plates and flasks were obtained from Costar (Cambridge, MA). Trypsin-EDTA, antibiotic/antimycotic and fetal bovine serum (FBS) were ordered from Gibco (Invitrogen Canada, Burlington, ON). High performance thin layer chromatography plates were purchased from Whatman Inc. (Clifton, NJ, USA). Earle's and Hank's minimum essential media and all other chemicals were purchased from Sigma (St. Louis, MO) and were declared to be cell culture grade and high purity.

4.2.2 Cell culture

The human colon cancer cell line, CaCo-2, was obtained from American Type Culture Collection (Manassas, VA) and was grown (passage 50-65) under standard incubator conditions (humidified atmosphere, 5% CO_2, 37°C) as adherent monolayer cultures in T-flasks containing Earle's minimum essential medium (EMEM). The cell culture growth medium (pH 7.2) was supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) antibiotic/antimycotic, 26 mM sodium bicarbonate, 10 mM HEPES and 1 mM pyruvic acid. Media was changed every 2-3 days and cell density and viability was assessed with a hemocytometer using the trypan blue dye exclusion method.

4.2.3 Isolation and purification of gangliosides

Total lipids were extracted from a GD3-enriched zeta dairy lipid powder. In short, zeta lipid 2 was dissolved in chloroform/methanol 2:1 (v/v) and homogenized. After vortexing, the tubes were placed on a shaker overnight and ddH_20 was added to give a 5:1 ratio of chloroform/methanol 2:1 (v/v) to ddH_20. To extract gangliosides, the ganglioside containing upper aqueous phase was collected and the lower organic phase was washed twice with Folch upper phase solution (chloroform/methanol/water, 3/48/47 by volume). The pooled upper phase ganglioside containing fractions were purified by passage through pre-washed Sep-Pak C_{18} reverse-phase cartridges (Waters Corporation, Milford, MA, USA). Once the upper phase extract was loaded onto the column and washed with ddH_20, gangliosides were eluted from the column with methanol and chloroform/methanol 2:1 (v/v). The eluate was dried under N_2 gas (Prax Air, Mississauga, Ontario) and the ganglioside powder re-dissolved in chloroform/methanol 2:1 (v/v). Gangliosides were stored at -20°C.

Individual gangliosides were separated on silica gel high performance thin layer chromatography plates (HPTLC; Whatman Inc., Clifton, NJ) with ganglioside standards in a solvent system of chloroform/methanol/ammonium hydroxide/water (60:35:7:3, v/v). To visualize ganglioside bands, HPTLC plates were sprayed with 0.1% (w/v) ANSA (8-anilino-1-napthalene-sulfonic acid) and viewed under ultraviolet light. The GD3 ganglioside bands were scraped off HPTLC plates and pooled together. GD3 was extracted from silica using the same method as ganglioside extraction from zeta lipid

powder. Extracted GD3 was purified, eluted, dried and re-dissolved as previously described.

Gangliosides were isolated from CaCo-2 cells by sonication in Tris-mannitol lysis buffer and extraction in chloroform/methanol (2:1, v/v). Extraction and purification was completed as described previously.

4.2.4 Intestine cell sensitivity to gangliosides

Human intestine CaCo-2 cells were seeded at a density of 400,000 cells/ml on a 24 well plate. At confluence, cells were switched into EMEM-4 (4% FBS) and treated with a range of GD3 concentrations (0-64 μg/ml) similar or higher than the amount in human milk on the apical side for 24 h under standard incubator conditions. Cell necrosis was accessed using a lactate dehydrogenase (LDH) release cytotoxicity assay kit (Promega, Madison, WI). In brief, cell lysates and supernatants were incubated with an enzyme/substrate solution for 30 min at room temperature and the absorbance of colored formazan product was measured at 490 nm with a microtiter plate reader (Molecular Devices, Sunnyvale, CA). Absorbance background reading under kit conditions was less than 0.075. Data were expressed as percentage LDH release from cells. The concentration of GD3 chosen for absorption experiments was based on intestine cell sensitivity to gangliosides.

4.2.5 Ganglioside uptake by human intestine cells

Polarized, differentiated monolayers of CaCo-2 cells grown on trans-well plates were used as a model to study apical and basolateral GD3 uptake (Figure 4-1).

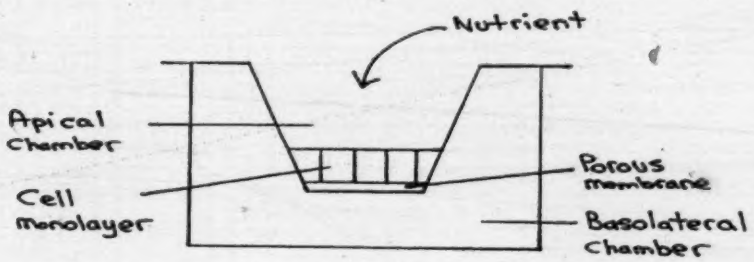


Figure 4-1 Schematic of intestinal epithelial monolayers grown on trans-well plates

CaCo-2 cells were seeded at a density of $2.5x10^7$ cells/ml on 0.4 μm pore inserts in 24 well trans-well plates (Corning Inc., Acton, MA). The apical (upper) and basolateral (lower) compartments contained 100 μl and 600 μl of EMEM-10, respectively. CaCo-2 cells were grown to confluence and were differentiated twenty days prior to delivering gangliosides. After 20 days, CaCo-2 cells were differentiated and polarized with intact tight junctions, as confirmed by alkaline phosphatase activity and trans-epithelial resistance measurements (data not shown). On day 21, CaCo-2 cells were transferred into Hank's MEM-4, a low serum, low calcium medium containing no antibiotics to limit serum and calcium interference with ganglioside uptake and antibiotic effects on tight junction permeability. Serum present in the media aided in GD3 solubilization and micelle formation during sonication. CaCo-2 cells were incubated under standard conditions in the absence (control, MEM-4 alone) or presence of purified ganglioside GD3 in MEM-4 for 6, 24 and 48 h at a concentration of 5 or 10 μg/ml. The time frame for exposing human intestine CaCo-2 cells to ganglioside GD3 was chosen to be 6-48 h as previous work on ganglioside GM1 uptake by cultured cells predicts slow and incomplete ganglioside absorption from the apical side[39,40]. The GD3 concentration chosen for absorption experiments is physiological; close to the concentration of gangliosides in mature human milk (9.07 μg lipid bound sialic acid per ml) and high enough to exert biological effects without exhibiting toxicity[41,42]. The ganglioside treated cells received GD3 on either the BBM (apical) side or the BLM side of CaCo-2 cell monolayers. GD3 delivery on the apical and BLM side of CaCo-2 cells was used as an in vitro model for enteral and intravenous nutrient delivery, respectively. After GD3 incubation, apical and BLM supernatants were collected and cells were washed with PBS and Tris buffer containing EDTA. CaCo-2 cells from each trans-well insert were trypsinized, pooled together in Tris-mannitol buffer and sonicated to form cell lysates. Cell lysates were saved for protein and ganglioside analysis.

4.2.6 Determination of cell protein

The amount of protein in cell lysates was determined using the bicinchoninic acid (BCA) assay. Cell lysates were diluted 1 in 4 with ddH_2O. Bovine serum albumin standards and diluted cell lysates (10 μl) were each mixed with 190 μl of a 50:1 mixture

of BCA solution and 4% $CuSO_4 \cdot 5H_2O$ for 30 min at 37°C. The absorbance at 562 nm was measured with a microplate reader (Molecular Devices Co., USA).

4.2.7 Ganglioside analysis

Total and individual gangliosides were measured with a colorimetric sialic acid assay as ganglioside bound N-acetyl neuraminic acid (GG-NANA). An aliquot of purified ganglioside sample was dried under N_2 gas and dissolved with 0.5 ml of ddH_20 and 0.5 ml of resorcinol-HCl in screw-capped Teflon-lined tubes. The purple-blue color developed by reacting resorcinol reagent with ganglioside sialic acid at 160°C for 8 min was extracted into butylacetate/butanol (85:15, v/v) solvent. Optical density was read by a spectrophotometer at 580 nm. For quantitative analysis, N-acetyl neuraminic acid (also known as sialic acid) was used as a standard.

4.2.8 Calculating uptake, retention, transfer and metabolism of ganglioside GD3

The following equations were used to calculate the percentage and amount of ganglioside GD3 taken up, retained, transferred and metabolized by human intestinal CaCo-2 cells following brush border membrane or basolateral membrane GD3 delivery. For all calculations the values obtained from the control were subtracted off the test groups to correct for small levels of ganglioside present in the serum and cells.

Equation 1 $$\% \text{ uptake} = \frac{(\text{amt delivered} - \text{amt remaining})}{\text{amt delivered}} * 100\%$$

Equation 2 $$\% \text{ retention} = \frac{\text{amt cell}}{\text{amt uptake}} * 100\ \%$$

Equation 3 $$\% \text{ transfer} = \frac{\text{amt receiving compartment}}{\text{amt uptake}} * 100\%$$

Equation 4 $$\% \text{ metabolism} = \frac{\text{amt uptake} - \text{amt retained} - \text{amt transferred}}{\text{amt uptake}} * 100\%$$

4.2.9 Statistical Analysis

Sample size determination for a one tailed comparison at 80% power to detect a 4-5 fold increase in GM1 levels in skin fibroblasts (0.2 nmol/mg protein to 0.9 nmol/mg protein, $P< 0.05$) requires a sample size of 3[40]. All data are expressed as the mean ± the standard error of the mean for a sample size of n=4 (4 different cell passages) for intestine cell sensitivity experiments and n=3-4 (3-4 different cell passages) for ganglioside GD3 absorption experiments. Significant differences between the control and experimental group were determined by a one way analysis of variance (ANOVA) and a Tukey test using SAS statistical software version 9.1. Differences between all treatments were considered statistically significant at an alpha of $P<0.05$.

4.3 RESULTS

4.3.1 Sensitivity of human intestinal CaCo-2 cells to ganglioside GD3

To determine the GD3 concentration range that does not exhibit toxicity to the enterocyte for absorption experiments, human intestinal CaCo-2 cells were exposed to a range of ganglioside GD3 concentrations (0-64 μg/ml) for 24 h and cell necrosis was assessed by measuring percentage lactate dehydrogenase (LDH) release from cells. Exposure of CaCo-2 cells to 64 μg/ml GD3 induced significant release of LDH from cells (**Fig. 4-2**). Although GD3 was not tested at higher concentrations, a crude mixture of gangliosides containing 80% GD3 continues to induce enterocyte toxicity at concentrations as high as 775 μg/ml although the mixture was less potent than pure GD3 at the same concentration (data not shown). Knowing that the concentration of gangliosides in mature human milk is 9.07 μg of lipid bound sialic acid per ml[41] and in human serum is 10.5 nmol of lipid bound sialic acid per ml[43], a concentration of 10 μg/ml was chosen as the physiological concentration of GD3 for absorption experiments. A concentration of 10 μg/ml exerts anti-inflammatory effects on infant bowel without exhibiting toxicity (Chapter 5).

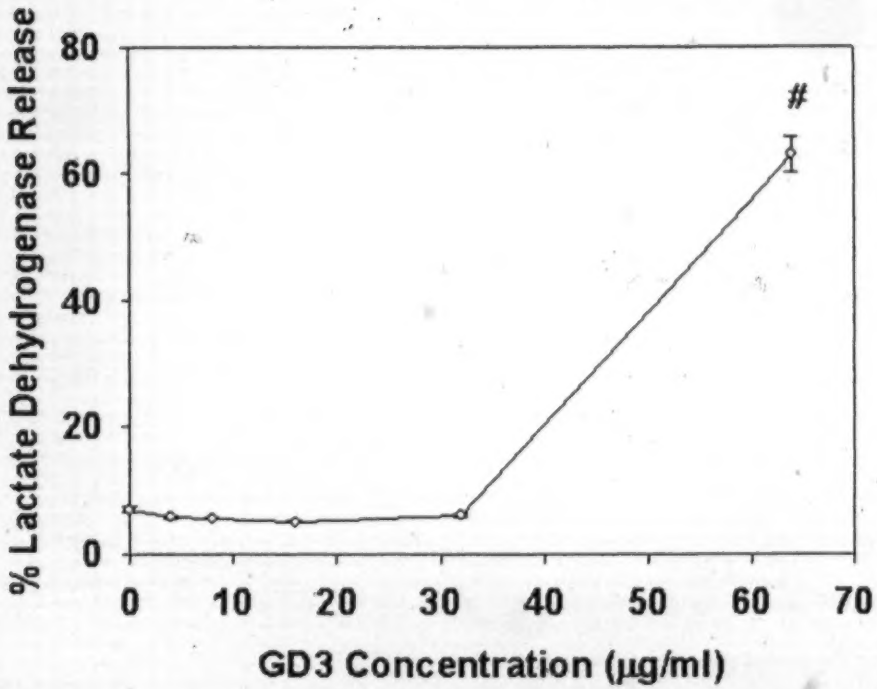


Fig. 4-2 Sensitivity of human intestinal CaCo-2 cells to apical ganglioside GD3. Human intestinal CaCo-2 cells were exposed to a range of GD3 concentrations for 24 h and the percentage release of lactate dehydrogenase from cells was measured to access cell membrane integrity. Ganglioside GD3 did not affect the osmolality of culture media. Data are expressed as the mean ± the standard error of the mean for four different cell passages (n = 4). # $P < 0.0001$ compared to all other concentrations of ganglioside GD3.

4.3.2 Time-dependent uptake of ganglioside GD3 by human intestinal CaCo-2 cells

Human intestinal CaCo-2 cells were exposed to 10 µg/ml ganglioside GD3 on the apical and basolateral membrane side for 6, 24 and 48 h at 37°C, to determine if uptake of exogenous ganglioside GD3 is time-dependent. The time course of GD3 uptake was rapid and linear for the first 6 h, slowed from 6 h to 48 h and reached a plateau (**Fig. 4-3**). Ganglioside GD3 uptake was faster and more efficient when GD3 was delivered on the basolateral membrane side of CaCo-2 cell monolayers when compared to apical delivery (**Fig. 4-3**).

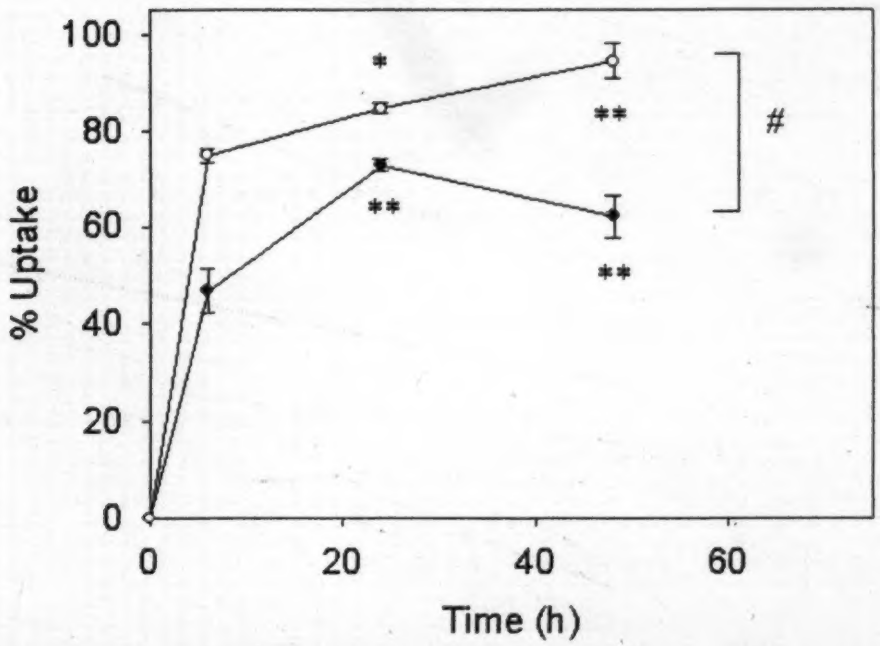


Figure 4-3 Percentage uptake of ganglioside GD3 into human intestinal CaCo-2 cells over time following addition of 10 μg/ml ganglioside GD3 to the apical side (•) or basolateral membrane (BLM) side (◦) of human intestinal CaCo-2 cell monolayers. Each data point represents the mean ± the standard error of the mean for three different cell passages (n = 3). * $P < 0.05$ compared to uptake at 6 h; ** $P < 0.005$ compared to uptake at 6 h and # $P < 0.0001$ difference between apical and BLM uptake at all measured time points.

4.3.3 Concentration dependent uptake of ganglioside GD3 by human intestinal CaCo-2 cells

Human intestinal CaCo-2 cells were exposed to 5 or 10 μg/ml ganglioside GD3 on the basolateral membrane side for 6 h at 37°C, to determine if uptake of exogenous ganglioside GD3 is concentration-dependent. The amount of ganglioside GD3 taken up from the basolateral membrane side by human intestinal CaCo-2 cell monolayers was twice has high when the concentration of GD3 added was doubled (**Fig. 4-4**).

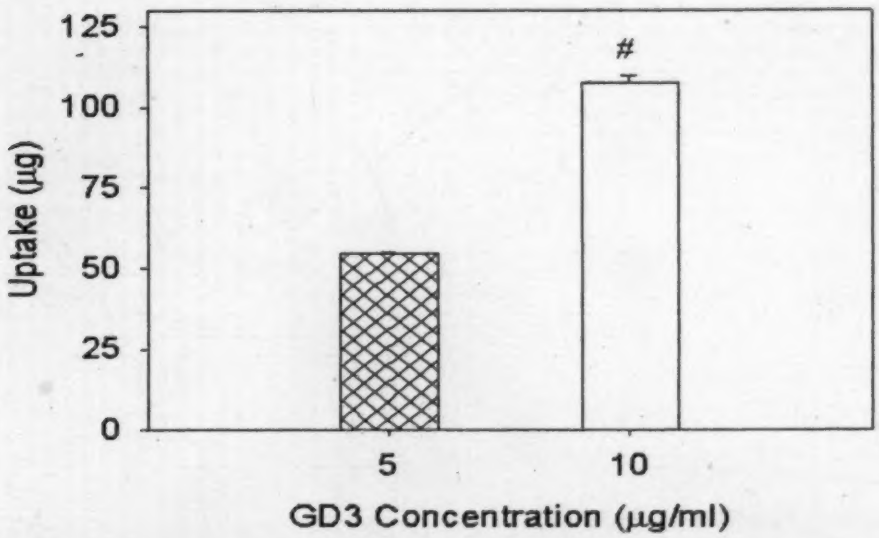


Figure 4-4 Amount of ganglioside GD3 uptake into human intestinal CaCo-2 cells after 6 h exposure to 5 µg/ml or 10 µg/ml ganglioside GD3 delivered on the basolateral membrane side of human intestinal CaCo-2 cell monolayers. Each bar represents the mean ± the standard error of the mean for three different cell passages (n = 3). # $P<$ 0.0001.

4.3.4 Fate of ganglioside GD3 taken up by human intestinal CaCo-2 cells

Changes in cellular retention, metabolism and transfer of ganglioside GD3 taken up by human intestinal CaCo-2 cells over time was followed to determine the fate of GD3 taken up from the brush border membrane and basolateral membrane side. The amount of GD3 taken up from the BBM side was not significantly higher at 24 or 48 h when compared to 6 h (**Table 4-1**). After 6 h apical exposure to GD3, 96% percent of GD3 taken up was metabolized with small amounts retained in the cell membrane and trace amounts transferred across the basolateral membrane (**Table 4-1**). After 24 h apical exposure to GD3, the percentage GD3 metabolized dropped 32% and significantly larger amounts of GD3 were retained in the cell membrane and transferred across the basolateral membrane (**Table 4-1**). The amount of GD3 taken up from the BLM side was significantly higher at 24 h and 48 h when compared to 6 h (**Table 4-2**). Similar to apical GD3, basolateral membrane GD3 uptake following 6 h exposure was almost completely destined for metabolism with trace amounts retained in the cell

membrane or transferred across the apical membrane (**Table 4-2**). In contrast to apical GD3, basolateral membrane GD3 uptake following 24 h exposure was completely metabolized with significant turnover of cell membrane GD3 and transfer of small insignificant amounts of GD3 across the apical membrane (**Table 4-2**).

Table 4-1 Uptake, retention, transfer and metabolism of apical ganglioside GD3 by human intestine CaCo-2 cells over time.

Apical (BBM)	6 h	24 h	48 h
Uptake (μg)	11±1.1	19±1.1	15±1.0
Retention (%)	3.6±1.1	15±2.4#	9.6±0.9#
Retention (μg)	0.4±0.1	2.9±0.6**	1.3±0.2
Transfer (%)	1.6±0.04	20±4.3#	-4.2±1.1*
Transfer (μg)	0.2±0.01	3.6±0.5**	-0.6±0.3
Metabolism (%)	96±1.1	64±1.5#	91±1.0
Metabolism (μg)	11±0.5	12±0.9	13±1.2

Human intestinal CaCo-2 cells were exposed to 10 μg/ml GD3 for 6, 24 and 48 h on the brush border membrane (BBM) side of cell monolayers. For BBM delivery, 24 μg of GD3 was delivered to the top compartment of 24 pooled trans-wells (1 μg/100 μl/trans-well). Values are expressed as the mean ± the standard error of the mean for three different cell passages (n = 3). * $P < 0.05$; ** $P < 0.01$ and # $P \leq 0.001$ compared to 6 h.

Table 4-2 Uptake, retention, transfer and metabolism of basolateral ganglioside GD3 by human intestine CaCo-2 cells over time.

Basolateral (BLM)	6 h	24 h	48 h
Uptake (μg)	108±2.12	122±1.52#	136±5.33#
Retention (%)	1.53±0.35	-1.11±0.71	0.42±0.11
Retention (μg)	1.65±0.39	-1.35±0.88#	0.58±0.17
Transfer (%)	1.74±0.46	2.52±1.17	1.32±0.18
Transfer (μg)	1.69±0.44	3.06±1.40	1.78±0.18
Metabolism (%)	96.7±0.37	98.6±1.64	98.3±0.19
Metabolism (μg)	104±2.89	120±2.52#	134±5.30#

Human intestinal CaCo-2 cells were exposed to 10 μg/ml GD3 for 6, 24 and 48 h on the basolateral membrane (BLM) side of cell monolayers. For BLM delivery, 144 μg of GD3 was delivered to the bottom compartment of 24 pooled trans-wells (6 μg/600 μl/trans-well). Values are expressed as the mean ± the standard error of the mean for three different cell passages (n = 3). # $P \leq 0.001$ compared to 6 h.

4.4 DISCUSSION

Using human intestine CaCo-2 cell monolayers grown on trans-well inserts as a model for enteral and intravenous ganglioside absorption, this study identifies the physiological concentration range of ganglioside GD3 for the enterocyte and reveals the time frame, efficiency, mechanism and fate of GD3 uptake from the apical and basolateral side of enterocytes.

Human intestine CaCo-2 cell sensitivity experiments with a range of ganglioside GD3 concentrations demonstrated that ganglioside GD3 begins to exhibit toxicity to enterocytes after 24 h exposure at concentrations greater than 32 μg/ml (**Fig. 4-2**). Knowing that the concentration of gangliosides in mature human milk is 9.07 μg of lipid bound sialic acid per ml[41], ganglioside GD3 may be safely administered enterally at concentrations several times higher than that present in human milk to access nutrient kinetics and distribution in animals and humans. This study also suggests that a stronger therapeutic effect in the absence of toxicity may potentially be achieved when GD3 is administered at concentrations greater than 9 and less than 32 μg/ml.

This study is the first to examine the time frame and efficiency for apical ganglioside GD3 uptake. The results of this study demonstrate that apical GD3 uptake by human enterocytes begins rapidly within 6 h and reaches a plateau after 24 h. The drop in apical GD3 uptake after 48 h may be explained by disruption of ganglioside concentration gradients by ganglioside shedding as 10 μg/ml GD3 is not toxic to other epithelial cell lines after 48 or 72 h (data not shown). Dietary sulfatide, an acidic glycosphingolipid, has been shown to be primarily absorbed in the rat ileum with small amounts incorporated in 2 h, larger amounts incorporated in 6 h and most of the sulfatide disappearing from the intestine by 24 h[44]. When applied for 24-72 h at 37°C, ganglioside GM1 incorporates into fibroblasts in the range of a few nanomoles per mg cellular protein[39,40]. Compared to GM1 uptake studies using other cell lines, the time frame for GD3 uptake by intestine cells was similar, however, in contrast to GM1, GD3 incorporated in the range of a few micrograms per mg cell protein. Given that the amount of GM1 given to cells was similar or higher (1-150 μg/ml) than the amount of GD3 used in this absorption study (5-10 μg/ml) may suggest that GD3 is more bioavailable than GM1[35,39,40,45]. Uptake efficiency of ganglioside is influenced by

ganglioside structure, formulation, route of delivery, serum, temperature, pH, divalent cations and cell type, morphology and density[35]. Temperature, pH and cell density were fixed for GD3 absorption experiments while serum and divalent cation levels were kept low. In this absorption study, apical GD3 uptake efficiency of intestine cells reached a maximum of 73% and incorporated 18.5 μg of GD3 after 24 h. In contrast to the intestine, Giardia cells exposed to the same amount of ganglioside GM1 under similar experimental conditions reached a maximal uptake efficiency of only 10% and incorporated 2.4 μg of GM1 after 2 h[45]. Ganglioside GM1 is a microbial receptor and modulates ion-sensitive surface proteins while GD3 is implicated in intracellular signal regulation[25,45]. Based on ganglioside function, it seems reasonable that most GM1 should remain near the cell membrane surface for membrane protection while most GD3 should be taken up into the intestine cell to alter cell signaling. In translating these findings to clinical use, it is important to consider that in vitro systems provide estimates of uptake and may over-estimate since they are isolated systems that do not account for possible ganglioside losses due to gastric acidity, luminal secretions of digestive enzymes and gastrointestinal motility. A small amount of serum was included in the media so lipids and bile salts were present to aid in ganglioside solubilization and micellar formation. An apical ganglioside uptake efficiency of 73% by intestine cells in vitro seems reasonable as dietary cholesterol and sphingomyelin, other amphiphilic lipids, have apical uptake efficiencies of 50% and 22-43%, respectively for rat intestine in vivo[46,47]. The study shows that apical GD3 uptake by intestine cells is time-dependent, fast and efficient with clinical potential to enterally deliver GD3 to the intestine and perhaps other tissues via the circulation.

The time frame, concentration-dependence and efficiency for ganglioside GD3 uptake from the basolateral side of intestine have not been investigated. To date there is one human study on the pharmacokinetics of intravenous ganglioside GM1. Peak plasma concentrations of GM1 in healthy men were reached within 1 h after a 100, 200 or 300 mg intravenous bolus[48]. The peak plasma concentration attained was dose-dependent[48]. The plasma half life for GM1 was reported to be 8-12 h and it took up to 7 days for GM1 levels to reach steady state[48]. The time course for plasma GM1 fit a multi-compartment model with distinct redistribution and elimination phases[48]. Since

the volume of distribution was not calculated, it is not known whether most GM1 remained in the blood or whether some GM1 was distributed to tissues[48]. Renal excretion of GM1 was absent and tissue and lipoprotein GM1 was not measured[48]. Trace amounts of GM1 were present in the feces and the authors suggested that the small amount of GM1 in feces was from cell turnover and the diet[48]. Based on intravenous ganglioside kinetic studies in rats, the plasma half life for GM1 and GM3 is 1.4 h and 1.8 h, respectively, with plasma gangliosides reaching steady state in 12-24 h[49]. The shorter plasma half life is attributed to the rat HDL cycle; rats have higher HDL levels[49]. Since gangliosides are preferentially distributed within LDL and HDL over albumin and other lipoproteins the amount of gangliosides available for tissue uptake from the blood may be less in rats compared to humans. In rats, 75% of the radiolabel for GM1 and 38% of the radiolabel for GM3 were present in the liver after 3 h[49]. Most of the GM1 was metabolized to GM2 and GM3 while most of the GM3 remained intact[49]. Tissue distribution analysis revealed that 19% of GM1 radiolabel and 9.4% of GM3 radiolabel localized to the kidney, lung and brain[49]. This study suggests that at most 6% of GM1 and 47.4% of GM3 could have been distributed to the intestine. In this study, basolateral GD3 uptake was linear and rapid for the first 6 h then slowed down from 6-48 h. It can be anticipated that GD3 uptake levels off at a later time point as the percentage GD3 uptake approaches 100%. As the GD3 concentration was increased from 5 to 10 μg/ml on the basolateral side, twice as much GD3 was taken up by human intestine cells indicating that GD3 uptake is concentration-dependent. Moss[50] demonstrated that incorporation of gangliosides into other cell lines is also concentration-dependent. In contrast to the human GM1 study, basolateral GD3 uptake by intestine cells occurs faster than GM1 uptake/removal from the blood. Moreover, basolateral GD3 uptake was faster and 22% more efficient than apical GD3 uptake by human intestine cells. This finding is expected as CaCo-2 cells express digestive enzymes for glycosphingolipids within the brush border membrane that may not be present in the basolateral membrane[51-53]. Although intestine cells are effective at taking up basolateral GD3, an in vivo study would be required to assess the distribution preference of GD3 for other tissues, lipoproteins and metabolism. Similarity in the chemical structure of GM3 and GD3, aside from the additional sialic acid, may indicate

a similar kinetic and distribution profile. This study suggests that basolateral GD3 uptake by intestine cells is time-dependent, concentration-dependent, fast and efficient with clinical potential to deliver GD3 to the intestine intravenously.

Few studies have followed the uptake of a fixed ganglioside concentration over time. In this study, both apical and basolateral membrane uptake of GD3 followed second order kinetics and reached a plateau suggesting the presence of a carrier or passive ganglioside uptake dependent upon a concentration gradient. In the GM1 uptake study with Giardial trophozoites, GM1 uptake was linear, slowed after 80 min and reached a plateau at 120 min although a later time point could have been included to confirm that a steady state had been reached[45]. Since the plateau could indicate passive uptake of ganglioside GD3, further research is required to confirm that ganglioside uptake is carrier-mediated and/or energy-dependent.

Several lines of evidence suggest that the uptake of gangliosides into the cell membrane involves action of some specific proteins. Gangliosides added exogenously to epithelial cell cultures are taken up by the apical membranes but do not pass the tight junction to the basolateral membranes of the cell[54]. Pre-treatment of cells with trypsin reduces ganglioside uptake[55] and the recovery of ganglioside uptake ability requires de novo synthesis of proteins[56]. Finally, ganglioside shedding from membranes is an active process that appears to be regulated indicating specific protein activity[57].

Several proteins have been implicated in the binding and transfer of sphingolipids at the cell membrane surface including prosaposin, glycolipid transfer protein, lipoprotein receptors and several proteins involved in cholesterol absorption[13,58-61]. Prosaposin is the precursor protein of sphingolipid activator proteins and some prosaposin is retained at the outer side of cell membranes in association with gangliosides[13]. In vitro, prosaposin promotes transfer of gangliosides from liposomes to membranes in a concentration-dependent manner and catalyzes ganglioside transfer between different vesicles[13]. Both gangliosides and prosaposin function through the formation and modulation of lipid rafts, thus it has been hypothesized that a possible function of prosaposin in the cell membrane is to regulate the formation and modulation of lipid rafts by insertion or removal of specific gangliosides[13]. Although prosaposin is present in neural cell membranes, it is not known whether prosaposin is present in the

brush border or basolateral membrane of enterocytes[13]. Glycolipid transfer protein (GLTP) is a soluble protein that selectively accelerates intermembrane transfer of glycolipids in vitro[13]. More recently, proteins with similar activities have been found in a wide variety of tissues including brain, liver and kidney[13]. Interestingly, glycolipid transfer protein transfers glycolipids with shorter sugars more efficiently[13], which is consistent with the observation that GD3 and GM3 appear to be taken up more readily than GM1 which contains 2 additional sugar residues. On the basis of currently published results, prosaposin and GLTP are good candidate proteins involved in regulating the uptake and shedding of gangliosides.

There is also accumulating evidence suggesting that lipoprotein receptors may play a role in glycolipid uptake. Rensen[62] demonstrated that lipoproteins are loaded with glycolipids and that the recognition and uptake of lipoproteins by tissues was dependent on the dose of glycolipid incorporated into the lipoprotein. Sulfatides are acidic glycosphingolipids similar to gangliosides and have been shown to inhibit scavenger receptor uptake of LDL by macrophages[63]. Lipopolysaccharide is a glycolipid that when injected intravenously into experimental animals binds quickly to circulating lipoproteins and slowly clears from the circulation by tissues that express lipoprotein receptors[64]. Sphingomyelin has amphiphilic properties similar to gangliosides with a total of 63-75% of plasma sphingomyelin found in LDL and VLDL and 25-37% found in HDL[59]. Some sphingomyelin carried in lipoproteins is transferred to cells primarily via scavenger receptor class B type I and to a lesser extent LDL receptor[59]. Scavenger receptors class B type I are expressed at high levels in the intestine including CaCo-2 cells[65] and may be responsible in part for uptake of gangliosides from the apical and basolateral side of intestine.

Two other groups of cholesterol transporters have been implicated in the transport of sphingolipids, the Niemann-Pick C1 like 1 protein (NCP1L1) and the family of ATP-binding cassette (ABC) proteins. The NCP1L1 protein is an endosomal-lysosomal glycoprotein involved in the cellular transport of glycosphingolipids and cholesterol from endosomes to lysosomes[61]. Recently, expression of NCP1L1 was demonstrated in the human small intestine and CaCo-2 cells[66]. The protein was most highly expressed in microvilli of the jejunum luminal plasma membrane and co-

localized with SR-BI and ABC cholesterol transporters[66]. Knock out of the NCP1L1 protein reduced micellar uptake of lipids[66]. Of the ABC transporter family, ABCA1 and multi-drug resistance protein P glycoprotein (MDR1 PgP) are most likely to be involved in sphingolipid uptake and transfer. ABCA1 is mainly expressed in the liver, small intestine and placenta and is involved in ATP-dependent export of cholesterol, phospholipids and other lipophilic molecules into HDL[67]. Since many plasma gangliosides are carried in HDL the ABCA1 transporter appears to be a possible ganglioside transport candidate. The MDR1 PgP is found in the apical membrane of mucosal cells and the luminal membrane of endothelial cells and has been shown to translocate hydrophobic drugs, sphingomyelin and glucosylceramide, one of the precursors for ganglioside biosynthesis[60]. In summary, there are a large number of potential proteins involved in ganglioside transport necessitating further research in this growing area.

The fate of gangliosides taken up by human intestine has not been studied. According to Pagano[37] gangliosides taken up by cells have three fates: incorporation into the plasma membrane (possible storage pool), degradation by lysosomes and ganglioside modification in the Golgi. Other possible fates for gangliosides include turnover of intestinal epithelial cells sloughed off into the lumen, ganglioside shedding, chemical modification in the plasma membrane and transfer of gangliosides across the intestinal membrane[14]. To access the fate of ganglioside GD3 taken up by human intestine cells, the amount of GD3 retained in the cell, transferred across the membrane and metabolized by intestine cells was calculated. In this study, we demonstrate that apical GD3 was mostly metabolized after 6 h with small amounts retained by enterocytes or transferred across the basolateral membrane. After 24 h, the additional GD3 taken up by cells was retained or transferred resulting in a significant drop in the percentage of GD3 targeted for metabolism. Thus, apical GD3 that was taken up by intestine cells was prioritized to meet the needs of the enterocyte first. GD3 may be metabolized to release bioactive metabolites or may be incorporated into the plasma membrane as an intact or modified form. Once the needs of the enterocyte were met, any extra GD3 that entered the intestine cell was transferred across the basolateral membrane for transport in the circulation to other tissues. In contrast to apical GD3,

basolateral GD3 was completely targeted for metabolism, likely into ceramide, sphingosine or a free fatty acid. Extra GD3 taken up by enterocytes was not retained in the cell but metabolized with trace amounts moving across the apical membrane. Metabolism and removal of extra GD3 by enterocytes may be a protective mechanism since high levels of GD3 trigger apoptosis[32]. Alternatively, cellular GD3 may have been acetylated. Acetylation has recently been shown to be a mechanism that tumor cells use as protection from apoptosis[32]. Since the assay used for ganglioside analysis in this study measures sialic acid any GD3 that was acetylated at the sialic acid residue would have been classified as being metabolized. The influence of route of delivery on fate of GD3 may be explained by the distinct protein and lipid composition of the brush border and basolateral membrane. The brush border membrane is enriched in lipid rafts, organized regions within the membrane that are enriched in cholesterol, glycosphingolipids and signaling proteins[23]. Research demonstrates that gangliosides may be taken up by cells via endocytosis in lipid rafts and targeted to different intracellular pools by caveosomes which can by-pass lysosomal degradation[68]. Moreover, glycosphingolipids have been found in chylomicrons and may pass through chylomicron formation pathways targeted for transfer of glycosphingolipids into plasma lipoproteins[59]. The basolateral membrane does not contain lipid rafts but has abundant lipoprotein receptors. LDL uptake occurs via clathrin-dependent receptor mediated endocytosis, a pathway that leads to lysosomal degradation[69]. Thus, some gangliosides may by-pass metabolism when delivered on the apical side of the enterocyte. The difference in GD3 fate following uptake from different sides of the intestine cell suggests that route of delivery may be an important factor to consider when using gangliosides for different therapeutic purposes.

A limitation of this study is that lipid bound sialic acid was used as a measure of ganglioside GD3. Therefore, any modifications made to the sialic acid molecules such as acetylation of sialic acid that could not be accounted for would have been included as metabolized GD3. Moreover, due to the small amounts of gangliosides in cells it was not possible to distinguish between the different ganglioside structures. Furthermore, gangliosides are susceptible to degradation and we could not distinguish intact versus modified ganglioside. Ideally, a triply radiolabeled ganglioside in

combination with a sensitive analytical technique such as tandem mass spectrometry would overcome such challenges.

In summary, this study demonstrates that ganglioside GD3 uptake by human intestine cells is time-dependent, concentration-dependent and efficient when delivered on both the apical and basolateral side of enterocytes. Evidence is provided to suggest that ganglioside uptake may involve a carrier or concentration gradient with intracellular concentrations of GD3 tightly regulated by the enterocyte. Furthermore, the study indicates that route of delivery is important to consider when using GD3 for therapeutic purposes as the uptake efficiency and fate differs depending on the route. Further work is required to identify enterocyte carriers for ganglioside uptake and transport. A more detailed assessment of fate is necessary to clarify how much ganglioside is chemically modified versus intact. Finally, an in vivo study in animals with a similar blood lipoprotein composition as humans is required to assess the kinetics, tissue distribution and safety of intravenous GD3 prior to a clinical pharmacokinetic study in humans.

4.5 REFERENCES

1. Farhadi, A., Banan, A., Fields, J. & Keshavarzian, A. Intestinal barrier: an interface between health and disease. *J Gastroenterol Hepatol* 18, 479-97 (2003).
2. Martel, F., Goncalves, P. & Azevedo, I. Absorption of folate by Caco-2 cells is not affected by high glucose concentration. *Eur J Pharmacol* 551, 19-26 (2006).
3. Reboul, E. et al. Effect of the main dietary antioxidants (carotenoids, gamma-tocopherol, polyphenols, and vitamin C) on alpha-tocopherol absorption. *Eur J Clin Nutr* (2007).
4. Chantret, I., Barbat, A., Dussaulx, E., Brattain, M.G. & Zweibaum, A. Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of twenty cell lines. *Cancer Res* 48, 1936-42 (1988).
5. Fogh, J., Fogh, J.M. & Orfeo, T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* 59, 221-6 (1977).
6. Chantret, I. et al. Differential expression of sucrase-isomaltase in clones isolated from early and late passages of the cell line Caco-2: evidence for glucose-dependent negative regulation. *J Cell Sci* 107 (Pt 1), 213-25 (1994).
7. Harris, D.S., Slot, J.W., Geuze, H.J. & James, D.E. Polarized distribution of glucose transporter isoforms in Caco-2 cells. *Proc Natl Acad Sci U S A* 89, 7556-60 (1992).

8. Peterson, M.D., Bement, W.M. & Mooseker, M.S. An in vitro model for the analysis of intestinal brush border assembly. II. Changes in expression and localization of brush border proteins during cell contact-induced brush border assembly in Caco-2BBe cells. *J Cell Sci* 105 (Pt 2), 461-72 (1993).
9. Glahn, R.P., Cheng, Z., Welch, R.M. & Gregorio, G.B. Comparison of iron bioavailability from 15 rice genotypes: studies using an in vitro digestion/caco-2 cell culture model. *J Agric Food Chem* 50, 3586-91 (2002).
10. Okada, T., Narai, A., Matsunaga, S., Fusetani, N. & Shimizu, M. Assessment of the marine toxins by monitoring the integrity of human intestinal Caco-2 cell monolayers. *Toxicol In Vitro* 14, 219-26 (2000).
11. Pal, D., Udata, C. & Mitra, A.K. Transport of cosalane-a highly lipophilic novel anti-HIV agent-across caco-2 cell monolayers. *J Pharm Sci* 89, 826-33 (2000).
12. Walle, U.K. & Walle, T. Transport of the cooked-food mutagen 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine (PhIP) across the human intestinal Caco-2 cell monolayer: role of efflux pumps. *Carcinogenesis* 20, 2153-7 (1999).
13. Lauc, G. & Heffer-Lauc, M. Shedding and uptake of gangliosides and glycosylphosphatidylinositol-anchored proteins. *Biochim Biophys Acta* 1760, 584-602 (2006).
14. Tettamanti, G. Ganglioside/glycosphingolipid turnover: new concepts. *Glycoconj J* 20, 301-17 (2004).
15. Forstner, G.G. & Wherrett, J.R. Plasma membrane and mucosal glycosphingolipids in the rat intestine. *Biochim Biophys Acta* 306, 446-59 (1973).
16. Park, E.J. et al. Diet-induced changes in membrane gangliosides in rat intestinal mucosa, plasma and brain. *J Pediatr Gastroenterol Nutr* 40, 487-95 (2005).
17. Dahiya, R. & Brasitus, T.A. Distribution of glycosphingolipids and ceramide of rat small intestinal mucosa. *Lipids* 21, 112-6 (1986).
18. Clarke, J.T. The glycosphingolipids of human plasma lipoproteins. *Can J Biochem* 59, 412-7 (1981).
19. Rueda, R., Maldonado, J., Narbona, E. & Gil, A. Neonatal dietary gangliosides. *Early Hum Dev* 53 Suppl, S135-47 (1998).
20. Vesper, H. et al. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J Nutr* 129, 1239-50 (1999).
21. Park, E.J., Suh, M., Thomson, A.B., Ramanujam, K.S. & Clandinin, M.T. Dietary gangliosides increase the content and molecular percentage of ether phospholipids containing 20:4n-6 and 22:6n-3 in weanling rat intestine. *J Nutr Biochem* 17, 337-44 (2006).
22. Allende, M.L. & Proia, R.L. Lubricating cell signaling pathways with gangliosides. *Curr Opin Struct Biol* 12, 587-92 (2002).
23. Fantini, J., Maresca, M., Hammache, D., Yahi, N. & Delezay, O. Glycosphingolipid (GSL) microdomains as attachment platforms for host pathogens and their toxins on intestinal epithelial cells: activation of signal transduction pathways and perturbations of intestinal absorption and secretion. *Glycoconj J* 17, 173-9 (2000).

24. Hakomori, S. Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. *J Biol Chem* 265, 18713-6 (1990).
25. Ledeen, R.W. & Wu, G. Ganglioside function in calcium homeostasis and signaling. *Neurochem Res* 27, 637-47 (2002).
26. Merrill, A.H., Jr. et al. Sphingolipids--the enigmatic lipid class: biochemistry, physiology, and pathophysiology. *Toxicol Appl Pharmacol* 142, 208-25 (1997).
27. Berra, B. et al. Glycosphingolipid expression in solid tumours and transformed cell lines. *Indian J Biochem Biophys* 34, 170-7 (1997).
28. Draper, J.S., Pigott, C., Thomson, J.A. & Andrews, P.W. Surface antigens of human embryonic stem cells: changes upon differentiation in culture. *J Anat* 200, 249-58 (2002).
29. Morales, A., Colell, A., Mari, M., Garcia-Ruiz, C. & Fernandez-Checa, J.C. Glycosphingolipids and mitochondria: role in apoptosis and disease. *Glycoconj J* 20, 579-88 (2004).
30. Wang, J.H. et al. [Ganglioside GD3 enhances the anti-tumor effect of doxorubicin on hepatoma cells]. *Zhonghua Gan Zang Bing Za Zhi* 14, 710-1 (2006).
31. Malisan, F. & Testi, R. The ganglioside GD3 as the Greek goddess Hecate: several faces turned towards as many directions. *IUBMB Life* 57, 477-82 (2005).
32. Kniep, B. et al. 9-O-acetyl GD3 protects tumor cells from apoptosis. *Int J Cancer* 119, 67-73 (2006).
33. Popa, I. et al. Purification and structural characterization of de-N-acetylated form of GD3 ganglioside present in human melanoma tumors. *Glycobiology* 17, 367-73 (2007).
34. Bieberich, E. Integration of glycosphingolipid metabolism and cell-fate decisions in cancer and stem cells: review and hypothesis. *Glycoconj J* 21, 315-27 (2004).
35. Saqr, H.E., Pearl, D.K. & Yates, A.J. A review and predictive models of ganglioside uptake by biological membranes. *J Neurochem* 61, 395-411 (1993).
36. Schwarzmann, G. Uptake and metabolism of exogenous glycosphingolipids by cultured cells. *Semin Cell Dev Biol* 12, 163-71 (2001).
37. Pagano, R.E. Lipid traffic in eukaryotic cells: mechanisms for intracellular transport and organelle-specific enrichment of lipids. *Curr Opin Cell Biol* 2, 652-63 (1990).
38. Malinina, L. et al. The liganding of glycolipid transfer protein is controlled by glycolipid acyl structure. *PLoS Biol* 4, e362 (2006).
39. Albrecht, B., Pohlentz, G., Sandhoff, K. & Schwarzmann, G. Synthesis and mass spectrometric characterization of digoxigenin and biotin labeled ganglioside GM1 and their uptake by and metabolism in cultured cells. *Chem Phys Lipids* 86, 37-50 (1997).
40. Midorikawa, M. et al. Uptake and metabolism of radiolabelled GM1-ganglioside in skin fibroblasts from controls and patients with GM1-gangliosidosis. *J Inherit Metab Dis* 14, 721-9 (1991).

41. Bode, L., Beermann, C., Mank, M., Kohn, G. & Boehm, G. human and bovine milk gangliosides differ in their fatty acid composition. *J Nutr* 134, 3016-20 (2004).
42. Suh, M., Belosevic, M. & Clandinin, M.T. Dietary lipids containing gangliosides reduce Giardia muris infection in vivo and survival of Giardia lamblia trophozoites in vitro. *Parasitology* 128, 595-602 (2004).
43. Rebbaa, A. & Portoukalian, J. Distribution of exogenously added gangliosides in serum proteins depends on the relative affinity of albumin and lipoproteins. *J Lipid Res* 36, 564-72 (1995).
44. Blomqvist, M., Osterbye, T., Mansson, J.E., Buschard, K. & Fredman, P. Uptake of the glycosphingolipid sulfatide in the gastrointestinal tract and pancreas in vivo and in isolated islets of Langerhans. *Lipids Health Dis* 5, 26 (2006).
45. Pope-Delatorre, H., Das, S. & Irwin, L.N. Uptake of [3H]-gangliosides by an intestinal protozoan, Giardia lamblia. *Parasitol Res* 96, 102-6 (2005).
46. Altmann, S.W. et al. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* 303, 1201-4 (2004).
47. Schmelz, E.M., Crall, K.J., Larocque, R., Dillehay, D.L. & Merrill, A.H., Jr. Uptake and metabolism of sphingolipids in isolated intestinal loops of mice. *J Nutr* 124, 702-12 (1994).
48. Cornelli, U. et al. Pharmacokinetics of GM1 ganglioside following parenteral administration. *J Pharm Pharmacol* 42, 708-11 (1990).
49. Dumontet, C., Rebbaa, A. & Portoukalian, J. Kinetics and organ distribution of [14C]-sialic acid-GM3 and [3H]-sphingosine-GM1 after intravenous injection in rats. *Biochem Biophys Res Commun* 189, 1410-6 (1992).
50. Moss, J., Fishman, P.H., Manganiello, V.C., Vaughan, M. & Brady, R.O. Functional incorporation of ganglioside into intact cells: induction of choleragen responsiveness. *Proc Natl Acad Sci U S A* 73, 1034-7 (1976).
51. Liu, F., Cheng, Y., Wu, J., Tauschel, H.D. & Duan, R.D. Ursodeoxycholic acid differentially affects three types of sphingomyelinase in human colon cancer Caco 2 cells. *Cancer Lett* 235, 141-6 (2006).
52. Shen, Y., Tiralongo, J., Kohla, G. & Schauer, R. Regulation of sialic acid O-acetylation in human colon mucosa. *Biol Chem* 385, 145-52 (2004).
53. Sugawara, T., Kinoshita, M., Ohnishi, M., Nagata, J. & Saito, M. Digestion of maize sphingolipids in rats and uptake of sphingadienine by Caco-2 cells. *J Nutr* 133, 2777-82 (2003).
54. Spiegel, S., Blumenthal, R., Fishman, P.H. & Handler, J.S. Gangliosides do not move from apical to basolateral plasma membrane in cultured epithelial cells. *Biochim Biophys Acta* 821, 310-8 (1985).
55. Boltz-Nitulescu, G., Ortel, B., Riedl, M. & Forster, O. Ganglioside receptor of rat macrophages. Modulation by enzyme treatment and evidence for its protein nature. *Immunology* 51, 177-84 (1984).
56. Leskawa, K.C., Erwin, R.E., Leon, A., Toffano, G. & Hogan, E.L. Incorporation of exogenous ganglioside GM1 into neuroblastoma membranes: inhibition by calcium ion and dependence upon membrane protein. *Neurochem Res* 14, 547-54 (1989).

57. Ladisch, S., Gillard, B., Wong, C. & Ulsh, L. Shedding and immunoregulatory activity of YAC-1 lymphoma cell gangliosides. *Cancer Res* 43, 3808-13 (1983).
58. Milis, D.G., Moore, M.K., Atshaves, B.P., Schroeder, F. & Jefferson, J.R. Sterol carrier protein-2 expression alters sphingolipid metabolism in transfected mouse L-cell fibroblasts. *Mol Cell Biochem* 283, 57-66 (2006).
59. Nilsson, A. & Duan, R.D. Absorption and lipoprotein transport of sphingomyelin. *J Lipid Res* 47, 154-71 (2006).
60. Raggers, R.J., van Helvoort, A., Evers, R. & van Meer, G. The human multidrug resistance protein MRP1 translocates sphingolipid analogs across the plasma membrane. *J Cell Sci* 112 (Pt 3), 415-22 (1999).
61. Sagiv, Y. et al. Cutting edge: impaired glycosphingolipid trafficking and NKT cell development in mice lacking Niemann-Pick type C1 protein. *J Immunol* 177, 26-30 (2006).
62. Rensen, P.C., van Leeuwen, S.H., Sliedregt, L.A., van Berkel, T.J. & Biessen, E.A. Design and synthesis of novel N-acetylgalactosamine-terminated glycolipids for targeting of lipoproteins to the hepatic asialoglycoprotein receptor. *J Med Chem* 47, 5798-808 (2004).
63. Yoshiizumi, K., Nakajima, F., Dobashi, R., Nishimura, N. & Ikeda, S. Studies on scavenger receptor inhibitors. Part 1: synthesis and structure-activity relationships of novel derivatives of sulfatides. *Bioorg Med Chem* 10, 2445-60 (2002).
64. Munford, R.S. Detoxifying endotoxin: time, place and person. *J Endotoxin Res* 11, 69-84 (2005).
65. Bietrix, F. et al. Accelerated lipid absorption in mice overexpressing intestinal SR-BI. *J Biol Chem* 281, 7214-9 (2006).
66. Sane, A.T. et al. Localization and role of NPC1L1 in cholesterol absorption in human intestine. *J Lipid Res* 47, 2112-20 (2006).
67. Oram, J.F. & Vaughan, A.M. ATP-Binding cassette cholesterol transporters and cardiovascular disease. *Circ Res* 99, 1031-43 (2006).
68. Nichols, B. Caveosomes and endocytosis of lipid rafts. *J Cell Sci* 116, 4707-14 (2003).
69. Garuti, R. et al. The modular adaptor protein autosomal recessive hypercholesterolemia (ARH) promotes low density lipoprotein receptor clustering into clathrin-coated pits. *J Biol Chem* 280, 40996-1004 (2005).

CHAPTER 5 GANGLIOSIDES PROTECT BOWEL IN AN INFANT MODEL OF NECROTIZING ENTEROCOLITIS BY BALANCING VASOACTIVE AND INFLAMMATORY SIGNALS DURING LPS EXPOSURE AND HYPOXIA

5.1 INTRODUCTION

Necrotizing enterocolitis (NEC) is an inflammatory bowel disease of neonates and remains a gastrointestinal emergency in preterm infants as its current treatment does not target the underlying disease processes[1]. Although preterm birth, hypoxia-ischemia, infection and formula feeding are established risk factors [2]while feeding human milk is protective [3], the role of local vasoactive and inflammatory mediators in the underlying pathogenesis remains elusive due to limitations in current models. Existing models of NEC are limited in that these models use animals, immortalized cell lines or diseased tissue to study single risk factors [4-6]. Moreover, these models do not contain the aspect of prematurity that is the predisposing factor in NEC [7]. Previous studies have measured plasma or serum vasoactive and inflammatory mediators which reflect the systemic rather than the local intestinal response [7]. Several vasoactive and inflammatory mediators including endothelin-1 (ET-1), nitric oxide (NO), platelet activating factor (PAF), leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2), cytokines (tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12 (IL-12) and interleukin-18 (IL-18)) and reactive oxygen species (ROS) are considered to play synergistic and central roles in the inflammatory pathway leading to NEC [4,7-9]. Serotonin promotes bacterial translocation and is elevated during bowel inflammation, bowel obstruction and infection, but the role of serotonin in NEC has not been investigated [10-12].

Evidence suggesting that breast-fed infants are one tenth as likely to develop NEC as formula-fed infants led to the hypothesis that bioactive factors in human milk might be responsible for protective effects [3]. Gangliosides, sialic acid-containing glycosphingolipids, are located at the surface of cell membranes and in the milk fat globule membrane [13]. Evidence shows important roles for gangliosides in various cell functions by serving as: regulators of microbial attachment [14,15], membrane stabilizers [16], modulators of membrane bound or intracellular proteins including receptors, ion channels and enzymes[16], and precursors of intracellular signaling molecules involved in

cell growth, differentiation and immune responses [17,18]. Dietary gangliosides are taken up by the intestine, localize to different regions based on function and change the ganglioside content and composition of lipid rafts [19]. GD3 is the most abundant ganglioside in colostrum and neonatal bowel [15]. Recently, we demonstrated that a GD3 enriched ganglioside diet increases GD3 in the basolateral membrane, reduces PAF levels in intestinal lipid rafts and reduces pro-inflammatory eicosanoid and cytokine production in weanling rats infected with *E. coli* lipopolysaccharide (LPS) [20]. Therefore, we developed a human NEC model to study the role of local vasoactive and inflammatory mediators in the pathogenesis of the disease and the role of gangliosides in modulating the inflammatory response of infant bowel to LPS exposure and hypoxia. It was hypothesized that gangliosides would protect infant bowel from injury by restoring vasoactive and inflammatory mediator balance during LPS exposure and hypoxia. In this study, we identify serotonin and GM-CSF as mediators involved in the pathogenesis of NEC and show that gangliosides markedly reduce infant bowel necrosis by reducing local nitric oxide, endothelin-1, LTB_4, PGE_2, H_2O_2, IL-1β, IL-6 and IL-8 production in response to enterotoxic *E. coli* LPS and hypoxia. These findings reveal that gangliosides protect infant bowel by suppressing local inflammatory responses of infant bowel to both LPS exposure and hypoxia.

5.2 METHODS

5.2.1 Subjects and Bowel Tissue

The Health Ethics Research Board of the Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Canada approved the study and written informed consent was obtained from at least one parent. Nine infants between 28 and 42 weeks gestational age requiring open bowel surgery for intestinal atresias, and insertion or re-connection of jejuno/ileostomies were recruited from the neonatal intensive care unit at the Stollery Children's Hospital. Up to 1 cm of normally discarded stoma bowel and/or full thickness bowel were resected from close to the re-anastomosis site for tissue culture experiment. Two thirds of tissue samples were obtained from the ileum/proximal colon, regions most often associated with NEC. Pediatric surgeons minimized the tissue ischemic period to less than 1 min. Inflammatory or infectious

bowel, infants with Hirschsprung's disease and infant bowel with associated anomalies were excluded from the study.

5.2.2 Cell and Tissue Culture

Human intestine CaCo-2 cells were obtained from American Type Culture Collection (Manassas, Virginia, USA). Minimum essential media containing Earle's salts (EMEM) was used for human intestine CaCo-2 cell culture and was supplemented with 4.2 mM sodium bicarbonate, 10 mM HEPES, 1 mM pyruvic acid, 1% (v/v) antibiotic/antimycotic and 10% (v/v) fetal bovine serum. Human intestine CaCo-2 cells between passages 42-69 were used for experiments. CaCo-2 cells were grown in T75 flasks until confluence in a 37°C incubator with an atmosphere of 95% O_2/5% CO_2. For the oxygen consumption experiment, human intestine CaCo-2 cells (3.4×10^6 cells in 30 ml of EMEM-10) were grown for 4 days until confluence in T150 flasks and treated with 0.5 mg/ml filter sterilized sodium thioglycollate. Cell culture media was collected at 0, 6, 12 and 24 h for determination of dissolved oxygen.

. Minimum essential media containing Hank's salts (HMEM) was used for bowel transport and was supplemented with 4.2 mM sodium bicarbonate, 10 mM HEPES, 1 mM pyruvic acid and 2% (v/v) heat inactivated fetal bovine serum (HMEM-2, pH 7.4). Phosphate buffered saline supplemented with 1mM pyruvic acid and 2 mM glutamine (pH 7.4) was used as a wash buffer. Bowel transport media and wash buffer were placed in sterile, vacuum insulated thermos bottles and were bubbled on ice for 1 hour with carbogen (95% O_2/5% CO_2) prior to transport on ice to the pediatric surgery unit. Surgeons excised a 1 cm piece of bowel and minimized the ischemic time to < 1 min. Bowel tissue was washed three times with wash buffer to remove feces, blood and mucus. After washing, the tissue was cut into ten 0.5 cm x 0.5 cm pieces with sterile scissors. Each piece of tissue was placed mucosa up between two sterile pieces of gauze. The gauze was placed on the bottom of the thermos bottle containing bowel transport media. Following transport to the lab, the thermos containing bowel samples was put on ice and bubbled with carbogen. Each piece of tissue was weighed and pinned mucosa up onto a 12 well tissue culture plate containing 1.5 ml of bowel culture medium. Minimum essential medium containing Hank's salts was used for bowel

culture and was supplemented with 4.2 mM sodium bicarbonate, 10 mM HEPES, 1 mM pyruvic acid and 4% heat inactivated fetal bovine serum (HMEM-4, pH 7.4). One piece of bowel tissue was fixed with Bock's solution in a Histo Prep tissue cassette, washed with PBS and stored in 10% formalin for hematoxylin and eosin staining.

5.2.3 Oxygen Consumption

Percentage oxygen consumption was assayed using a biological oxygen monitoring system and oxygen probe (Yellow Springs Instrument Co, Ohio, USA) with high sensitivity membranes. Samples were stirred and maintained at 37°C during measurements.

5.2.4 Extraction, Purification, Analysis and Delivery of Gangliosides

Total lipids were extracted from zeta lipid-2 milk powder (Fonterra, Cambridge, New Zealand). In short, 0.5 g of lipid powder was dissolved in 30 ml of chloroform/methanol 2:1 (v/v) and homogenized for 30 s. After vortex, the tubes were placed on a shaker overnight. The following day 6 ml of ddH_20 was added to the tubes to give a 5:1 ratio of chloroform/methanol 2:1 (v/v) to ddH_20. To extract gangliosides, the ganglioside containing upper aqueous phase was transferred to a glass tube and the lower phase was washed twice with Folch upper phase solution (chloroform/methanol/water, 3/48/47 by volume). The pooled upper phase ganglioside containing fractions were purified by passage through Sep-Pak C18 reverse-phase cartridges (Waters Corporation, Milford, MA, USA) pre-washed with 10 ml methanol, 20 ml chloroform/methanol 2:1 (v/v) and 10 ml methanol. Once the upper phase extract was loaded on the column and washed with 20 ml of ddH_20, gangliosides were eluted from the column with 5 ml methanol and 20 ml chloroform/methanol 2:1 (v/v). The eluate was dried under N_2 gas (Prax Air, Mississauga, Ontario) and the ganglioside powder re-dissolved in 2 ml chloroform/methanol 2:1 (v/v). Gangliosides were stored in the -20°C freezer.

Total gangliosides were measured as ganglioside bound N-acetyl neuraminic acid (also known as sialic acid) as described previously [50]. An aliquot of purified ganglioside sample was dried under N_2 gas and dissolved with 0.5 ml ddH_20 and 0.5 ml

resorcinol-HCl in screw-capped Teflon-lined tubes. The purple- blue color developed by heating at 160°C for 8 min was extracted into butyl acetate/butanol (85:15, v/v) solvent. Optical density was read by a spectrophotometer at 580 nm. For quantitative analysis, N-acetyl neuraminic acid was used as a standard.

The ganglioside mixture used in experiments was similar to the composition of human colostrum, predominantly GD3 with smaller amounts of GM3, GM1 and GD1a. An aliquot of ganglioside stock dissolved in chloroform/methanol 2:1 (v/v) was dried under N_2 gas and dissolved in bowel culture medium (HMEM-4) to give a final concentration of 100 μg/ml. Ganglioside dissolution and micelle formation was facilitated by a 30 s sonication prior to filter sterilization of the ganglioside solution through a 0.2 μm pore syringe filter.

5.2.5 Infant Bowel Model of Necrotizing Enterocolitis

Infant bowel tissue was incubated at 37°C in an atmosphere of 95% O_2/5% CO_2 for 10 h in the presence or absence of 10 μg/ml ganglioside. After 10 h, the supernatant was collected and centrifuged at 14,000 rpm for 10 min to remove tissue debris. The clarified supernatant was transferred to sterile microcentrifuge tubes and stored in the -80°C freezer until analysis. Bowel tissue supernatant was replaced with HMEM-4 (control), 1 μg/ml Escherichia coli lipopolysaccharide (LPS treatment, serotype O111:B4), 0.5 mg/ml oxygen chelating sodium thioglycollate (hypoxia treatment) or LPS and sodium thioglycollate combined (NEC model) for 12 h at 37°C in an atmosphere of 95% O_2/5% CO_2. The concentrations of ganglioside, LPS and sodium thioglycollate used in the infant bowel model were established using CaCo-2 cell monolayers (data not shown). In anticipation that infant bowel would be more responsive to LPS than CaCo-2 cells, a lower concentration of LPS was chosen than what was effective in CaCo-2 cells (1 μg/ml instead of 50 μg/ml). After 12h, the supernatant was collected, clarified and stored as previously described. Bowel tissue was transferred with sterile forceps into sterile homogenization tubes on ice containing 1.5 ml of bowel culture medium (HMEM-4). Each piece of tissue was homogenized for 30 s and clarified by centrifuging at 3000 rpm, 4°C for 10 min. Supernatant from each

sample was aliquoted into microcentrifuge tubes prior to storage in the -80°C freezer to minimize sample freeze- thaw.

5.2.6 Tissue Viability and Integrity

Morphological examination of bowel tissue was achieved by placing a 0.5 cm section of bowel into a Histo Prep tissue cassette for fixation in Bock's solution and 10% (v/v) formalin. Following fixation, the tissue was sectioned into 5 μm thick pieces and stained with hematoxylin and eosin. Tissue samples with intact villi and absence of infiltrated immune cells were used in experiments. Any bowel tissue exhibiting signs of structural damage or inflammation did not culture well and was not included in experiments. The lactate dehydrogenase (LDH) in vitro toxicology kit (Promega) was used to assess cell necrosis as a function of the amount of cytoplasmic LDH released into bowel culture media. Following manufacturer directions, 50 μl of substrate mixture (enzyme in assay buffer) was added to 50 μl sample aliquots and the mixture was incubated for 30 min at room temperature. The reaction was stopped with 50 μl of acetic acid and the absorbance was measured at 490 nm using a spectrophotometer. Absorbance background under kit conditions was reported to be less than 0.075.

5.2.7 Vasoactive Mediator Production

Nitric oxide production was estimated by analyzing total nitrite (a by product of nitric oxide metabolism) concentration in bowel culture supernatants using a colorimetric assay kit based on the nitrate reductase and Griess reaction (Active Motif, California). In short, nitrate in samples was converted to nitrite in a 30 min nitrate reductase reaction. After the reaction, 100 μl of bowel culture supernatant was combined with 100 μl of Griess reagent (50 μl of 0.5% sulphanilamide in 6% phosphoric acid plus 50 μl of 0.05% naphthylethylene-diamine dihydrochloride in distilled water) and incubated for 10 min at room temperature. The absorbance was measured at 540 nm with a spectrophotometer and nitrite concentrations were determined with reference to a standard curve generated with sodium nitrite (0-100 μM) in bowel culture media. The detection limit for nitrite was 1 μM. Endothelin-1 levels in infant bowel lysates were measured with a commercially available enzyme-linked

immunosorbent assay (ELISA) kit (R & D Systems Inc., Minneapolis, MN). The absorbance was measured at 450 nm with a spectrophotometer and endothelin-1 concentrations were determined with reference to a standard curve generated with endothelin-1 standards (0-117 pg/ml). The detection limit for endothelin-1 was 1 pg/ml. Cross reactivity with endothelin-2 was reported to be 45%. Endothelin-2 is estimated to be present at less than 20% of the endothelin-1 level and is produced in smaller amounts during stress or disease compared to endothelin-1 [21]. Serotonin production was estimated by acylating serotonin in bowel culture supernatants and measuring N-acyl-serotonin concentration using a colorimetric competitive ELISA (Research Diagnostics Inc., New Jersey). The absorbance was measured at 405 nm with a spectrophotometer and N-acyl-serotonin concentrations were determined with reference to a standard curve generated with acylated serotonin standards (0-11 ng/ml). The detection limit for N-acyl-serotonin was 0.03 ng/ml.

5.2.8 Eicosanoid Extraction

Eicosanoids were extracted from bowel lysates by mixing an ethanol/water mixture (1:4 v/v) and 10 µl of glacial acetic acid with 500 µl of bowel lysate. The samples were centrifuged at 5000 rpm, 4°C for 5 min and loaded on to Sep Pak C18 cartridges pre-washed with 10 ml ddH_20, 10 ml 15% ethanol and 10 ml hexane. Eicosanoids were eluted off the column with 10 ml of ethyl acetate and dried under N_2.

5.2.9 Eicosanoid Analysis

Extracted eicosanoids were dissolved in 50 µl of 15% ethanol and 200 µl of bowel culture media. Leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) were measured in bowel lysates with established protocols provided with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN). The absorbance was measured at 450 nm with a spectrophotometer and eicosanoid concentrations were determined with reference to standard curves generated with LTB_4 and PGE_2 standards, respectively (0-3000 and 0-1250 pg/ml). The mean detection doses of LTB_4 and PGE_2 were 27.6 pg/ml and 10.8 pg/ml, respectively.

Cross-reactivity with other leukotriene and prostaglandin species was reported to be < 0.2% and < 17.5%, respectively.

5.2.10 Hydrogen Peroxide

The production of hydrogen peroxide by infant bowel was measured using a Quantichrom Peroxide Assay Kit (Bioassay Systems, California, USA). The optical density at 585 nm was measured with a spectrophotometer and the peroxide concentration of cell lysates was determined from a hydrogen peroxide standard curve. The detection limit for hydrogen peroxide was 14 ng/ml.

5.2.11 Cytokine Production

The IL-18 content of bowel lysates was measured with an ELISA kit (Biosource, Camarillo, California) using the manufacturer's protocol. The absorbance was measured at 450 nm and IL-18 concentrations were determined with reference to an IL-18 standard curve. A detection limit of 12 pg/ml was reported. The remaining mixture of pro-inflammatory cytokines, anti-inflammatory cytokines and TH_1/TH_2 cytokines including GM-CSF, IL-1β, TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-12 were measured in bowel lysates using the human cytokine 10 panel multiplex kit mixed with the IL-12 single plex kit. (Biosource, Camarillo, California). Multiplex cytokine assays were done on multi-screen 96 well filter plates that were covered with aluminum foil to minimize photo-bleaching. Assays were run in duplicate according to the manufacturer's protocol. To prevent well leakage, a blotting step for excess solution on the bottom of the filter plate was added after the final wash step before analysis. Data was collected using the Luminex 100 IS System and XY Platform (Applied Cytometry). Acquired fluorescence data were analyzed using Star Station software (Version Applied Cytometry). A five parameter weighted regression formula was used to calculate the sample concentrations from the standard curves. The detection limits for cytokines were between 3 and 15 pg/ml. Concentrations that fell below the detection limit were assigned values of half the detection limit.

5.2.12 Statistical Analysis

Sample size determination for a one-tailed comparison at 80% power to detect a 48% decrease in plasma LTB_4 levels (1354 pg/ml blood to 704 pg/ml blood, $P<0.05$) indicated that a sample size of 5 was required to demonstrate significance between control and treatment effects[22]. Due to variability between different samples and regions of intestine a larger sample size was required. All results were reported as the mean ± the standard error of the mean for a sample size of n = 3 (3 different cell passages) for the CaCo-2 cell experiment and n = 9 (9 different infant bowel samples) for the bowel culture experiment. The viability of cultured bowel tissue and distribution of immune cells within tissue samples was tested for normality by constructing a quantile-quantile plot with S-Plus. The plots for LDH and IL-8 release were approximately linear indicating that a normal distribution is a reasonable assumption for the data. Change in concentration of analyte between control and experimental treatments were analyzed with SAS using an analysis of variance and a Tukey test. A P value of less than 0.05 was considered statistically significant.

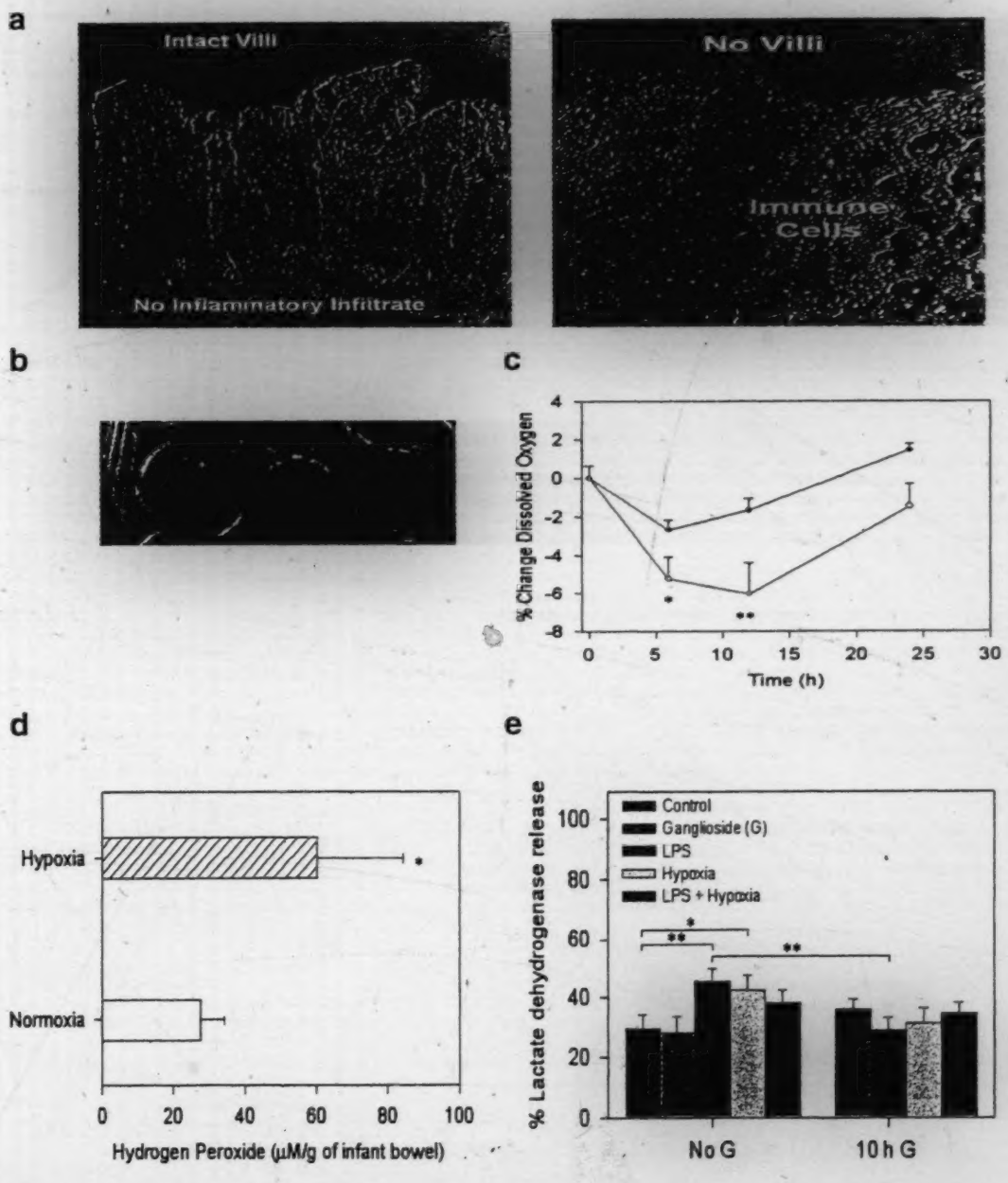
5.3 RESULTS

5.3.1 Gangliosides improve bowel survival during LPS exposure in an infant model of necrotizing enterocolitis

Since primary cultures of enterocytes have limited survival in culture [23], we developed a method to obtain viable, non-inflamed infant bowel during surgery (**Fig. 5-1a-b**). To overcome limitations of using *E. coli* LPS with immortalized intestinal cell lines and non-physiological hypoxia levels, we used enterotoxic *E. coli* LPS and an oxygen-chelating compound, sodium thioglycollate, to induce bowel necrosis and inflammation in cultured infant bowel as a model for NEC. Oxygen levels were 4.4% lower in media containing sodium thioglycollate after 12 h of human intestine cell culture and gradually returned to normal levels (**Fig. 5-1c**). Similar to other models of ischemia-reperfusion injury, sodium thioglycollate triggered production of reactive oxygen species (**Fig. 5-1d**). To test the model, we examined whether *E. coli* LPS and hypoxia induce necrosis in infant bowel. Using a lactate dehydrogenase (LDH) release assay to measure membrane integrity, we found that within 12 h of *E. coli* LPS or

Fig. 5-1 Development of an infant bowel model for necrotizing enterocolitis. **a,** Hematoxylin-eosin staining of healthy infant bowel (left) and necrotic bowel (right) obtained during intestinal surgery. Presence of intact villi and absence of infiltrated cells suggests infant bowel was viable and non-inflamed before culture. **b,** Full thickness infant bowel pinned mucosa up on a tissue culture plate containing bowel culture media. **c,** Human intestine CaCo-2 cells were grown in cell culture media with (°) or without (•) oxygen chelating sodium thioglycollate for 6, 12 and 24 h. The percentage change dissolved oxygen levels over time in cell supernatants is displayed in reference to baseline levels in cell culture media alone (Time=0 h). Dissolved oxygen levels were significantly lower in cell supernatants with sodium thioglycollate (°) than in cell supernatants without sodium thioglycollate (•) at 6 and 12 h. Data are mean ± the standard error of the mean for a sample size of n=3 (3 different cell passages). *P<0.01; **P<0.001. **d,** Infant bowel was cultured for 12 h with (hypoxia) or without (normoxia) sodium thioglycollate and H_2O_2 was measured in bowel lysates. Sodium thioglycollate significantly induced infant bowel production of H_2O_2. Data are mean ± the standard error of the mean for nine different infant bowel samples (n=9). *P<0.05. **e,** Infant bowel was exposed to *E. coli* LPS, hypoxia or the combination in the presence and absence of a 10 h ganglioside pre-exposure. Compared to the control, *E. coli* LPS and hypoxia induced significant bowel necrosis when gangliosides were not present as determined by loss of cell membrane integrity (lactate dehydrogenase release). Ganglioside pre-exposure reduced bowel necrosis following LPS treatment. Values represent mean ± the standard error of the mean for nine different infant bowel samples (n=9). *P<0.05, **P≤0.01.

hypoxia treatment, there was a marked increase in LDH release from infant bowel (**Fig. 5-1e**). Exposure of infant bowel to *E. coli* LPS with hypoxia did not significantly increase LDH release (**Fig. 5-1e**). To investigate whether gangliosides protect infant bowel from *E. coli* LPS- and hypoxia-induced necrosis, bowel tissue was pre-exposed to gangliosides for 10 h prior to treatments. The results show that gangliosides significantly attenuate *E. coli* LPS-induced release of LDH (**Fig. 5-1e**).



5.3.2 Gangliosides inhibit nitric oxide release from infant bowel during LPS exposure and hypoxia

The previous experiment indicates that *E. coli* LPS or hypoxia alone induce necrosis in infant bowel. Although the combination did not induce necrosis within 12 h, LPS and hypoxia could play an early, secondary role contributing to enterocyte apoptosis and inflammation. Inducible nitric oxide synthase (iNOS) levels are consistently elevated in models of NEC and toxic levels of NO are associated with enterocyte apoptosis and inflammation [9,24,25]. Thus, we postulated that *E. coli* LPS and hypoxia may trigger release of toxic concentrations of NO from infant bowel. To examine this hypothesis and determine the role of gangliosides in the process, total nitrite levels were measured in infant bowel treated with LPS, hypoxia or the combination in the presence and absence of a 10 h period of pre-exposure to ganglioside. The results demonstrate that *E. coli* LPS and hypoxia induce significant release of nitrite by infant bowel and that hypoxia enhances the effect of LPS on nitrite release (**Fig. 5-2**). When infant bowel was pre-exposed to gangliosides, nitrite release following *E. coli* LPS treatment in the presence and absence of hypoxia was abolished (**Fig. 5-2**).

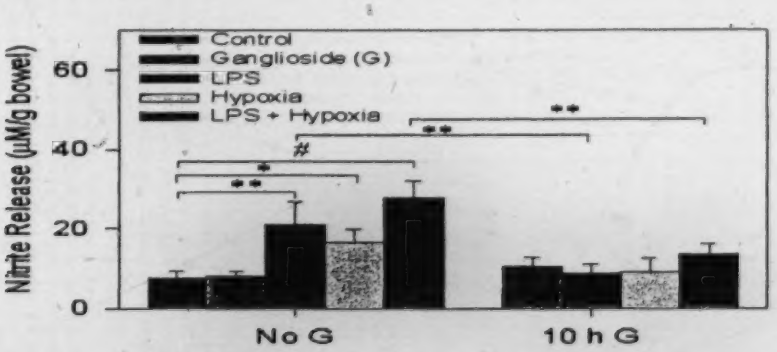


Fig. 5-2 Inhibitory effect of gangliosides on infant bowel production of nitric oxide during LPS exposure and hypoxia. Infant bowel was exposed to enterotoxic *E. coli* LPS, hypoxia or the combination in the presence and absence of a 10 h period of pre-exposure to ganglioside. When gangliosides where not present, LPS, hypoxia and the combined LPS and hypoxia treatment induced significant production of nitric oxide compared to the control as measured by total nitrite, the metabolic by product of nitric oxide. Ganglioside pre-exposure reduced nitric oxide release from infant bowel following enterotoxic *E. coli* LPS exposure and the combined LPS and hypoxia treatment. Values represent mean ± the standard error of the mean for nine different infant bowel samples (n=9). *P<0.05, **P<0.005, #P<0.0001.

5.3.3 Gangliosides reduce infant bowel production of vasoconstrictors, endothelin-1 and serotonin, during LPS exposure and hypoxia

Endothelin-1 is elevated in infant bowel with histopathological features of NEC and induces ischemia, hypoxia and tissue death [8]. Although serotonin reuptake inhibitors have been associated with some cases of NEC, the role of serotonin has not been investigated in this disease [26]. Thus, we postulated that *E. coli* LPS or hypoxia may trigger production of vasoconstrictors, endothelin-1 and serotonin, in infant bowel and that gangliosides may modulate the process. We found that *E. coli* LPS significantly induces endothelin-1 production in infant bowel while hypoxia triggers serotonin release (**Table 5-1**). Pre-exposing infant bowel to gangliosides prior to LPS treatment markedly lowered endothelin-1 production (**Table 5-1**). Reduction in serotonin release from infant bowel by gangliosides during hypoxia was not significant (**Table 5-1**).

Table 5-1 Endothelin-1 and serotonin production by infant bowel following exposure to enterotoxic *E. coli* LPS, hypoxia or the combination in the presence and absence of a 10 h period of pre-exposure to ganglioside

Treatments	Endothelin-1 ($pgml^{-1}g^{-1}$ of bowel)		Serotonin ($ngml^{-1}g^{-1}$ of bowel)	
	No G	10 h G	No G	10 h G
Control	14.6 ±1.44	11.1±1.88	549±164	803±291
LPS	32.3±7.02*	9.08±1.97**	1020±258	742±177
Hypoxia	22.1±4.21	12.0±3.84	1470±377*	1080±247
LPS + Hypoxia	24.5±5.69	12.5±3.74	885±228	833±178

Values represent the mean ± the standard error of the mean for nine different infant bowel samples (n=9). *P<0.005 compared to control without ganglioside. **P<0.0001 compared to LPS treated without ganglioside. G = ganglioside.

5.3.4 Gangliosides inhibit eicosanoid and hydrogen peroxide production by infant bowel during LPS exposure and hypoxia

Eicosanoids, leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2), and reactive oxygen species (ROS) are inflammatory mediators involved in the pathogenesis of NEC. Although it is well established that cyclooxygenase 2 (COX-2) mRNA is elevated in perforated bowel tissue from infants with NEC [27], LTB_4 and ROS levels have not been examined in infant bowel. Based on hypoxia-induced NEC models in young animals, intestinal LTB_4 and ROS levels are elevated with anti-oxidant enzyme

treatment reducing inflammatory mediator damage to intestine [28,29]. Therefore, we examined the effect of *E. coli* LPS and hypoxia on production of eicosanoids and H_2O_2 in infant bowel and determined whether gangliosides modulate the process. *E. coli* LPS and hypoxia stimulated production of LTB_4 and PGE_2 in infant bowel (**Fig. 5-3a-b**) while hypoxia induced H_2O_2 production (**Fig. 5-3c**). Pre-exposure of infant bowel to gangliosides 10 h prior to LPS exposure and hypoxia reduced LTB_4 and PGE_2 production by 50% (**Fig. 5-3a-b**). Infant bowel production of H_2O_2 during hypoxia was reduced 61% by pre-exposure to gangliosides (**Fig. 5-3c**).

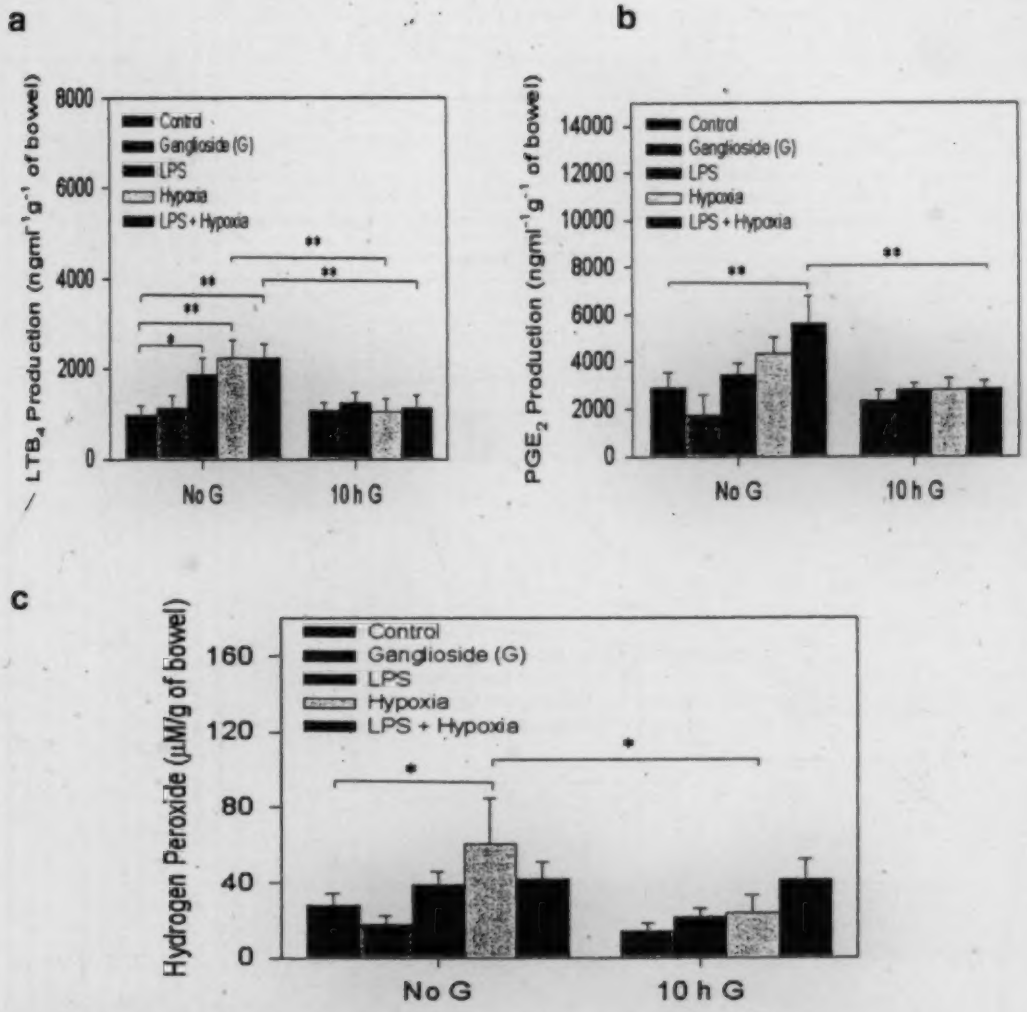
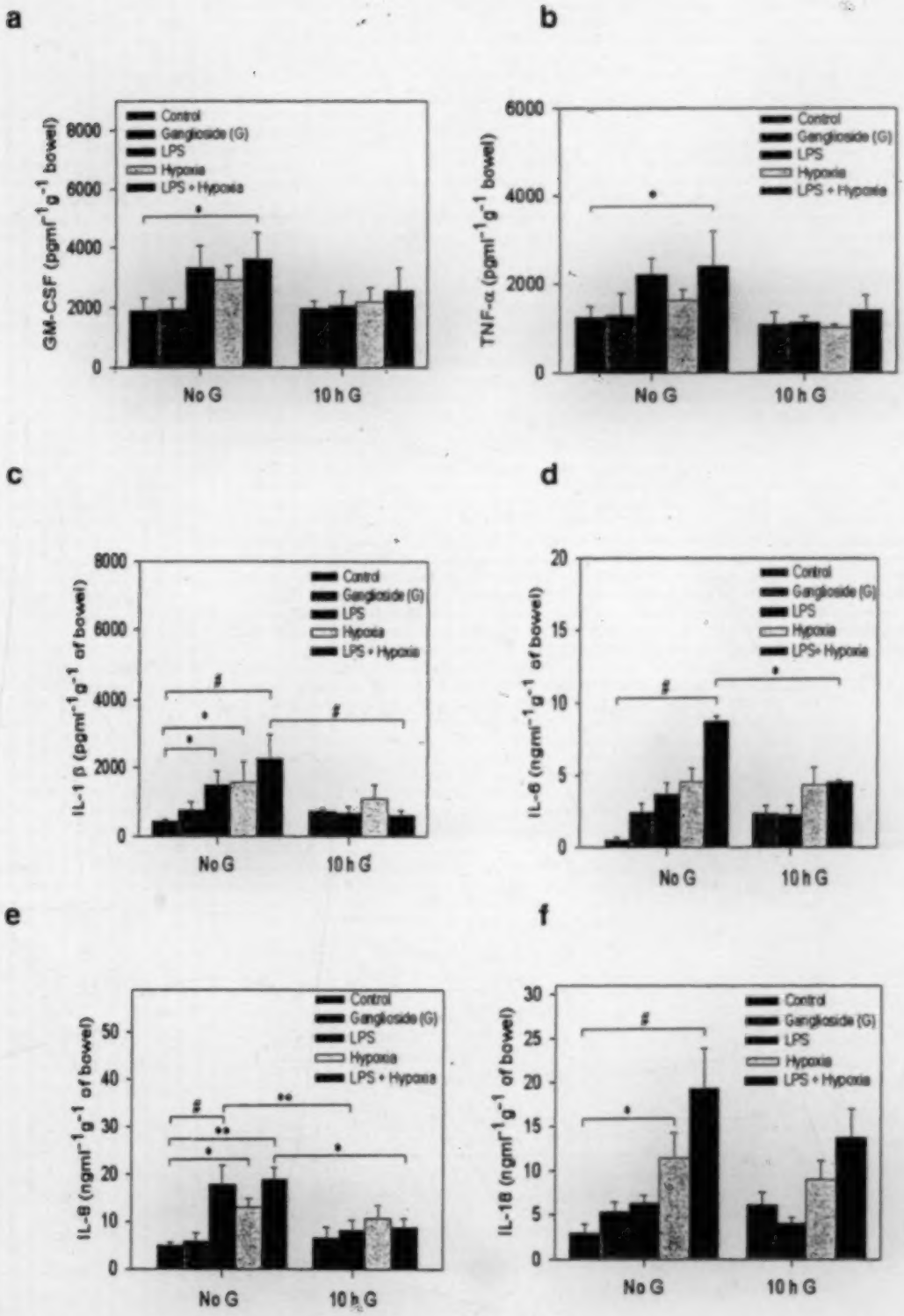


Fig. 5-3 Inhibitory effect of gangliosides on infant bowel production of pro-inflammatory eicosanoids and hydrogen peroxide during LPS exposure and hypoxia. Infant bowel was exposed to enterotoxic *E. coli* LPS, hypoxia or the combination in the presence and absence of a 10 h period of pre-exposure to ganglioside and LTB_4, PGE_2 and H_2O_2 were measured in bowel lysates. **a,** LTB_4. Compared to the control, LPS, hypoxia and the combined LPS and hypoxia treatment without gangliosides triggered significant production of LTB_4. Ganglioside pre-exposure reduced LTB_4 production by infant bowel following hypoxia and the combined LPS and hypoxia treatment. **b,** PGE_2. Compared to the control, the combined LPS and hypoxia treatment without gangliosides triggered significant production of PGE_2 by infant bowel. Ganglioside pre-exposure reduced PGE_2 production by infant bowel following combined LPS and hypoxia treatment. **c,** H_2O_2. Compared to the control, hypoxia triggered significant production of H_2O_2 by infant bowel. Ganglioside pre-exposure reduced infant bowel production of H_2O_2 following hypoxia. Values represent the mean ± the standard error of the mean for nine different infant bowel samples (n=9). *P<0.05, **P<0.01.

5.3.5 Gangliosides inhibit pro-inflammatory cytokine production in infant bowel during LPS exposure and hypoxia

The role of pro-inflammatory and anti-inflammatory cytokines in NEC remains elusive due to limitations in current models and analyses. Most studies have measured plasma and serum cytokines which reflect the systemic rather than the local intestinal response [7]. Measuring neonatal cell cytokines continues to be challenging as the neonate's immune system gives a low response to common mitogens. Evidence of local bowel cytokine production in NEC is limited to a few studies that measured cytokine mRNA expression in animals and in resected bowel tissue from infants with NEC [7]. Therefore, it was of interest to investigate the effect of *E. coli* LPS and hypoxia on production of pro-inflammatory, anti-inflammatory and TH_1/TH_2 cytokines in infant bowel tissue. For the first time, we show that granulocyte macrophage-colony stimulating factor (GM-CSF) is produced by infant bowel in response to *E. coli* LPS + hypoxia (**Fig. 5-4a**). Similar to other models of NEC, we demonstrate that *E. coli* LPS and hypoxia induce marked production of TNF-α, IL-1β, IL-6, IL-8 and IL-18 (**Fig. 5-4b-f**). Levels of IL-12, IFN-γ, IL-2, IL-4, IL-5 and IL-10 were below detection in control and treated bowel tissue. To investigate whether gangliosides exhibit anti-inflammatory properties, bowel tissue was pre-exposed to gangliosides for 10 h prior to treatments and pro-inflammatory cytokines were measured. The results show that gangliosides suppress infant bowel production of IL-1β, IL-6 and IL-8 during *E. coli* LPS treatment and hypoxia by 75%, 48% and 48%, respectively (**Fig. 5-4c-e**).

Fig. 5-4 Infant bowel pro-inflammatory cytokines involved in the pathogenesis of necrotizing enterocolitis and anti-inflammatory effects of gangliosides during LPS exposure and hypoxia. Infant bowel was exposed to *E. coli* LPS, hypoxia or the combination in the presence and absence of a 10 h period of pre-exposure to ganglioside and pro-inflammatory cytokines were measured in bowel lysates. **a,** Granulocyte macrophage-colony stimulating factor (GM-CSF). Compared to the control, the combined LPS and hypoxia treatment without gangliosides triggered significant production of GM-CSF by infant bowel. **b,** Tumor necrosis factor-alpha (TNF-α). The combined LPS and hypoxia treatment without gangliosides induced significant production of TNF-α by infant bowel in comparison to the control. **c,** Interleukin-1beta (IL-1β). Compared to the control, enterotoxic *E. coli* LPS, hypoxia and the combined LPS and hypoxia treatment without gangliosides induced significant production of IL-1β by infant bowel. Ganglioside pre-exposure reduced IL-1β production by infant bowel following the combined LPS and hypoxia treatment. **d,** Interleukin-6 (IL-6). The combined LPS and hypoxia treatment without gangliosides triggered significant production of IL-6 by infant bowel in comparison to the control. Ganglioside pre-exposure reduced IL-6 production by infant bowel following the combined LPS and hypoxia treatment. **e,** Interleukin-8 (IL-8). Compared to the control, enterotoxic *E. coli* LPS, hypoxia and the combined LPS and hypoxia treatment without ganglioside induced significant production of IL-8 by infant bowel. Ganglioside pre-exposure reduced IL-8 production by infant bowel following enterotoxic *E. coli* LPS and the combined LPS and hypoxia treatment. **f,** Interleukin-18 (IL-18). Compared to the control, hypoxia and the combined LPS and hypoxia treatment without gangliosides induced significant production of IL-18 by infant bowel. Values represent the mean ± the standard error of the mean for nine different infant bowel samples (n=9). *$P<0.05$, **$P<0.01$, $^{\#}P\leq0.0001$.


a
GM-CSF (pgml^{-1}g^{-1} bowel)
Control
Ganglioside (G)
LPS
Hypoxia
LPS + Hypoxia
No G
10 h G
b
TNF-α (pgml^{-1}g^{-1} bowel)
c
IL-1 β (pgml^{-1}g^{-1} of bowel)
d
IL-6 (ngml^{-1}g^{-1} of bowel)
e
IL-8 (ngml^{-1}g^{-1} of bowel)
f
IL-18 (ngml^{-1}g^{-1} of bowel)

5.4 DISCUSSION

An infant bowel model of NEC that overcomes limitations of current models was developed to investigate roles of local vasoactive and inflammatory mediators in the pathogenesis of NEC and protective effects of gangliosides. To overcome limited survival time of primary enterocyte cultures [23], a method to culture viable, non-inflamed infant bowel for 24 h was developed. Unlike immortalized intestinal cell lines that respond poorly to apical *E. coli* LPS [30], infant bowel produced significant amounts of vasoactive and pro-inflammatory mediators and developed necrosis following apical treatment with *E. coli* LPS. Instead of inducing acute intestinal injury using severe hypoxic-ischemic conditions observed in most animal models of NEC [4], the present model gradually lowers oxygen and then recovers oxygen to normal levels using sodium thioglycollate, an oxygen chelator that establishes anaerobic conditions for bacteria growth. Intestinal inflammation tends to be less severe after complete removal of oxygen as compared with gradual removal [4]. Due to absence of pressure flow autoregulation and poor responses to circulatory stress, neonatal intestine is sensitive to small changes in blood flow and oxygen. Hydrogen peroxide was generated during hypoxia suggesting that the model represents ischemia-reperfusion injury similar to other models of NEC. The novelty of the model is that it uses infant tissue and includes the aspect of immaturity that consistently predisposes neonates to this disease along with exposure to compound risk factors such as infection and hypoxia.

In the present model, enterotoxic *E. coli* LPS and hypoxia alone significantly induced lactate dehydrogenase release from infant bowel suggesting loss of membrane integrity and cell necrosis. The extent of tissue damage was not as profound as expected, particularly during combined LPS and hypoxia treatment, indicating that the model might represent an early point in disease progression where transition from apoptosis to necrosis is not complete. The time frame of 24 h in which viable infant bowel can be cultured and treated is limited by microbial growth. However, bowel necrosis was significant enough following LPS treatment to demonstrate that ganglioside pre-exposure reduces bowel necrosis.

To identify the time frame for disease progression, nitric oxide, a vasoactive and inflammatory mediator strongly correlated with enterocyte apoptosis in NEC [9,31], was

measured following infant bowel *E. coli* LPS exposure and hypoxia. Similar to other models of NEC, high concentrations of total nitrite were produced by infant bowel following treatment with *E. coli* LPS and hypoxia. Given that NO regulated pro-apoptotic gene BNIP3 and nitrosative stress are associated with enterocyte apoptosis in resected intestine from infants with NEC [31,32], LPS exposure and hypoxia may induce infant bowel apoptosis in our model. Since apoptosis has been demonstrated to initiate bowel necrosis in models of NEC [24], our model represents progression of NEC from early to late stage. Treatment with LPS was a stronger stimulus for NO release than hypoxia. Basal NO production by neonatal intestine during hypoxia is poor [33]. It is also possible that preconditioning occurred and hypoxia inducible factors were released to protect against further hypoxic insult [34]. Nitric oxide production was enhanced when *E. coli* LPS was given under hypoxic conditions. It is well established that LPS triggers NO production by inducible NOS through interaction with toll like receptor 4 (TLR4) and activation of the NFκβ pathway (**Fig. 5-5**) [35]. In addition to the NFκβ pathway, hypoxia triggers NO production by the constitutive, calcium dependent neuronal nitric oxide synthase (**Fig. 5-5**) [4,36]. Since ganglioside pre-exposure reduced bowel nitrite production during LPS exposure in the presence and absence of hypoxia but not with hypoxia alone, it is likely that gangliosides inhibit NO production primarily by inhibiting the TLR4-NFκβ pathway (**Fig. 5-5**). Studies investigating the NFκβ and TLR4 pathways have shown that GM1 ganglioside binds to LPS and reduces TLR4 binding [37] while GD3 ganglioside inhibits NFκβ activation [17]. Since the ganglioside mixture used was similar to human milk composition with GM1 present in lower amounts than GD3, it is expected that LPS binding and membrane stabilizing effects of GM1 would be less prominent than NFκβ inhibition by GD3.

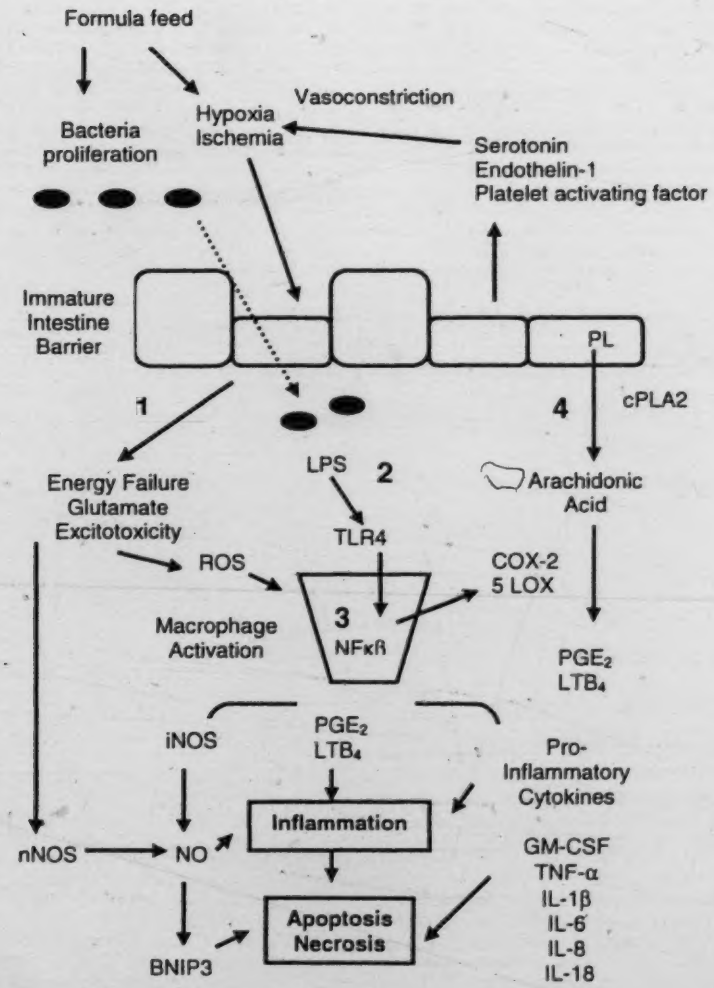


Fig. 5-5 Pathogenesis of necrotizing enterocolitis and potential mechanism by which gangliosides protect infant bowel from inflammation and injury. Risk factors associated with the development of necrotizing enterocolitis include pre-maturity, infection, hypoxia-ischemia and formula feeding. There are four possible mechanisms by which gangliosides protect infant bowel from injury: **1)** gangliosides stabilize membranes and reduce glutamate excitotoxicity during hypoxia; **2)** gangliosides bind LPS and prevent LPS interaction with toll like receptor 4 (TLR4); **3)** gangliosides inhibit NFκβ activation; and **4)** gangliosides inhibit cytosolic phospholipase A_2 ($cPLA_2$). PL = phospholipid, ROS = reactive oxygen species, COX-2 = cyclooxygenase-2, 5 LOX = 5 lipoxygenase, nNOS = neuronal nitric oxide synthase, iNOS = inducible nitric oxide synthase. BNIP3 = bcl-2 nineteen kilodalton interacting protein 3, GM-CSF = granulocyte macrophage-colony stimulating factor. **1, 2, 3, 4** = step inhibited by ganglioside

In addition to protecting infant bowel from apoptotic and necrotic injury, a homeostatic role for gangliosides in restoring vasoactive mediator balance was identified during LPS exposure and hypoxia. Nitric oxide and endothelin-1 balance is crucial for maintaining intestinal blood flow and this balance is disturbed in premature infants [8]. Endothelin-1 is also elevated in intestine afflicted with NEC and is associated with blood vessel constriction [8]. In the present model, NO and endothelin-1 levels are elevated during LPS exposure and hypoxia and restored to normal levels following pre-exposure to gangliosides. Since LPS induces endothelin-1 expression, it is speculated that gangliosides reduce endothelin-1 levels by binding to LPS and reducing LPS-mediated endothelin-1 synthesis [21] (**Fig. 5-5**). It is not known whether gangliosides interfere with endothelin-1 synthesis enzymes. The vasoconstrictor, serotonin, was also elevated during hypoxia in the present model, however, the reduction in serotonin following pre-exposure to gangliosides was not significant. It is not known whether gangliosides are taken up by serotonin-producing enterochromaffin cells but it is known that gangliosides alter serotonin receptor sensitivity [16] and interfere with downstream signaling pathways leading to serotonin induced vasoconstriction [38,39].

Gangliosides also exhibited both anti-inflammatory and anti-oxidant effects in the present model of NEC. During LPS treatment and hypoxia, pro-inflammatory eicosanoids, LTB_4 and PGE_2, involved in recruiting leukocytes to the inflammatory site were reduced by gangliosides. During hypoxia, gangliosides reduced infant bowel generation of H_2O_2, a reactive oxygen mediator that induces oxidative cell membrane damage and enterocyte apoptosis in NEC [40]. Eicosanoids are produced following NFκβ activation and during hypoxia, production is dependent upon calcium and reactive oxygen species produced following ischemia-reperfusion injury [36] (**Fig. 5-5**). Calcium and oxygen sensitive enzymes including cytosolic phospholipase A_2 ($cPLA_2$), 5-lipoxygenase and cyclooxygenase-2 generate LTB_4 and PGE_2 [36] (**Fig. 5-5**). Since gangliosides have been demonstrated to inhibit $cPLA_2$ [41] and enhance catalase activity [42], we propose that gangliosides exert anti-inflammatory and anti-oxidant effects by inhibiting NFκβ and $cPLA_2$ activation and by increasing catalase activity.

On the basis of cytokine findings, insight into the role of local cytokines in the pathogenesis of NEC was gained and anti-inflammatory properties of gangliosides were

reinforced in infant bowel. Granulocyte macrophage-colony stimulating factor (GM-CSF), a maturation, proliferation and activation factor of macrophages, was identified to be significantly elevated following *E. coli* LPS treatment and hypoxia. This is the first time GM-CSF has been associated with LPS exposure and hypoxia, possibly due to difficulty in measuring low plasma or serum GM-CSF levels. Neonatal immune cells produce low amounts of GM-CSF and GM-CSF receptor expression is reduced on neonatal immune cells [43]. In some of our bowel samples, GM-CSF and TNF-α were below detection limits. Our GM-CSF finding also raises concern in using GM-CSF to treat neutropenia in neonates at risk of developing NEC [44]. Although plasma IL-18 is not elevated in babies with NEC [45], we show that neonatal intestine IL-18 levels are increased following *E. coli* LPS exposure and hypoxia as shown previously in neonatal rat intestine [46]. The observation that ganglioside pre-exposure did not significantly reduce intestine IL-18 levels during LPS exposure and hypoxia may be explained by IL-18 polymorphism [47]. The basal level of intestinal IL-18 levels was highly variable between bowel samples from different subjects. In contrast to controversial NEC plasma cytokine studies, we clearly demonstrate that TNF-α, IL-1β, IL-6 and IL-8 are elevated in infant bowel following LPS exposure and hypoxia as seen in NEC resected infant bowel and neonatal rat intestine [7]. Ganglioside pre-exposure significantly prevented infant bowel production of pro-inflammatory cytokines IL-1β, IL-6 and IL-8. The anti-inflammatory effect is likely attributed to both the ability of the ganglioside to bind LPS and to directly prevent NFκβ activation (**Fig. 5-5**). We were unable to detect IL-12, IL-4, IL-10 and IFN-γ in all human neonatal bowel samples. Immature intestine has a reduced capacity to produce IFN-γ and IFN-γ and IL-12 mRNA have been reported as low in neonatal rat intestine afflicted with NEC [48,49]. IL-10 has been reported to reduce NEC symptoms and be elevated in late stage NEC [7,50]. Taken together with our observations, we propose that infant bowel has limited ability to produce IL-4, IL-10, IL-12 and IFN-γ under normal, infection and hypoxic conditions and that IL-10 and IL-12 production may be low in infant bowel in early stages of NEC.

In conclusion, we cultured human infant bowel and developed a physiological model that better represents the multifactorial, progressive nature of necrotizing enterocolitis in immature intestine. The data derived from studies in our NEC model,

identifies a role for GM-CSF and serotonin in the pathogenesis of NEC and a bowel protective effect of gangliosides through pro-inflammatory signal suppression, homeostatic control of vasoactive mediators regulating blood flow and anti-oxidant effects. The regulatory role of gangliosides in maintaining bowel survival and function demonstrated during LPS exposure and hypoxia provides a strong rationale for ganglioside use in food products to assess ganglioside safety and efficacy in promoting infant gut development and treating inflammatory bowel diseases.

5.5 REFERENCES

1. Panigrahi, P. Necrotizing enterocolitis: a practical guide to its prevention and management. *Paediatr Drugs* **8**, 151-65 (2006).
2. Horton, K.K. Pathophysiology and current management of necrotizing enterocolitis. *Neonatal Netw* **24**, 37-46 (2005).
3. Lucas, A. & Cole, T.J. Breast milk and neonatal necrotising enterocolitis. *Lancet* **336**, 1519-23 (1990).
4. Hsueh, W. et al. Neonatal necrotizing enterocolitis: clinical considerations and pathogenetic concepts. *Pediatr Dev Pathol* **6**, 6-23 (2003).
5. Nanthakumar, N.N., Fusunyan, R.D., Sanderson, I. & Walker, W.A. Inflammation in the developing human intestine: A possible pathophysiologic contribution to necrotizing enterocolitis. *Proc Natl Acad Sci U S A* **97**, 6043-8 (2000).
6. Sigge, W., Wedel, T., Kuhnel, W. & Krammer, H.J. Morphologic alterations of the enteric nervous system and deficiency of non-adrenergic non-cholinergic inhibitory innervation in neonatal necrotizing enterocolitis. *Eur J Pediatr Surg* **8**, 87-94 (1998).
7. Markel, T.A. et al. Cytokines in necrotizing enterocolitis. *Shock* **25**, 329-37 (2006).
8. Nowicki, P.T. et al. Endothelin-1 in human intestine resected for necrotizing enterocolitis. *J Pediatr* **146**, 805-10 (2005).
9. Ford, H.R. Mechanism of nitric oxide-mediated intestinal barrier failure: insight into the pathogenesis of necrotizing enterocolitis. *J Pediatr Surg* **41**, 294-9 (2006).
10. Linden, D.R. et al. Serotonin transporter function and expression are reduced in mice with TNBS-induced colitis. *Neurogastroenterol Motil* **17**, 565-74 (2005).
11. Wheatcroft, J. et al. Enterochromaffin cell hyperplasia and decreased serotonin transporter in a mouse model of postinfectious bowel dysfunction. *Neurogastroenterol Motil* **17**, 863-70 (2005).
12. Yamada, T., Inui, A., Hayashi, N., Fujimura, M. & Fujimiya, M. Serotonin stimulates endotoxin translocation via 5-HT3 receptors in the rat ileum. *Am J Physiol Gastrointest Liver Physiol* **284**, G782-8 (2003).
13. Tettamanti, G. Ganglioside/glycosphingolipid turnover: new concepts. *Glycoconj J* **20**, 301-17 (2004).

14. Fantini, J., Maresca, M., Hammache, D., Yahi, N. & Delezay, O. Glycosphingolipid (GSL) microdomains as attachment platforms for host pathogens and their toxins on intestinal epithelial cells: activation of signal transduction pathways and perturbations of intestinal absorption and secretion. *Glycoconj J* **17**, 173-9 (2000).
15. Rueda, R., Maldonado, J., Narbona, E. & Gil, A. Neonatal dietary gangliosides. *Early Hum Dev* **53** Suppl, S135-47 (1998).
16. Ledeen. (1989).
17. Morales, A., Colell, A., Mari, M., Garcia-Ruiz, C. & Fernandez-Checa, J.C. Glycosphingolipids and mitochondria: role in apoptosis and disease. *Glycoconj J* **20**, 579-88 (2004).
18. Vazquez, E., Gil, A. & Rueda, R. Dietary gangliosides positively modulate the percentages of Th1 and Th2 lymphocyte subsets in small intestine of mice at weaning. *Biofactors* **15**, 1-9 (2001).
19. Park, E.J. et al. Diet-induced changes in membrane gangliosides in rat intestinal mucosa, plasma and brain. *J Pediatr Gastroenterol Nutr* **40**, 487-95 (2005).
20. Park, E.J. et al. Dietary ganglioside decreases cholesterol content, caveolin expression and inflammatory mediators in rat intestinal microdomains. *Glycobiology* **15**, 935-42 (2005).
21. Wanecek, M., Weitzberg, E., Rudehill, A. & Oldner, A. The endothelin system in septic and endotoxin shock. *Eur J Pharmacol* **407**, 1-15 (2000).
22. Campbell, E.M. et al. Monocyte chemoattractant protein-1 mediates cockroach allergen-induced bronchial hyperreactivity in normal but not CCR2-/- mice: the role of mast cells. *J Immunol* **163**, 2160-7 (1999).
23. Rusu, D., Loret, S., Peulen, O., Mainil, J. & Dandrifosse, G. Immunochemical, biomolecular and biochemical characterization of bovine epithelial intestinal primocultures. *BMC Cell Biol* **6**, 42 (2005).
24. Jilling, T., Lu, J., Jackson, M. & Caplan, M.S. Intestinal epithelial apoptosis initiates gross bowel necrosis in an experimental rat model of neonatal necrotizing enterocolitis. *Pediatr Res* **55**, 622-9 (2004).
25. Di Lorenzo, M. & Krantis, A. Altered nitric oxide production in the premature gut may increase susceptibility to intestinal damage in necrotizing enterocolitis. *J Pediatr Surg* **36**, 700-5 (2001).
26. Stiskal, J.A., Kulin, N., Koren, G., Ho, T. & Ito, S. Neonatal paroxetine withdrawal syndrome. *Arch Dis Child Fetal Neonatal Ed* **84**, F134-5 (2001).
27. Chung, D.H. et al. Molecular mechanisms contributing to necrotizing enterocolitis. *Ann Surg* **233**, 835-42 (2001).
28. Akisu, M., Baka, M., Coker, I., Kultursay, N. & Huseyinov, A. Effect of dietary n-3 fatty acids on hypoxia-induced necrotizing enterocolitis in young mice. n-3 fatty acids alter platelet-activating factor and leukotriene B4 production in the intestine. *Biol Neonate* **74**, 31-8 (1998).
29. Miller, M.J., McNeill, H., Mullane, K.M., Caravella, S.J. & Clark, D.A. SOD prevents damage and attenuates eicosanoid release in a rabbit model of necrotizing enterocolitis. *Am J Physiol* **255**, G556-65 (1988).

30. Wells, C.L., Jechorek, R.P., Olmsted, S.B. & Erlandsen, S.L. Effect of LPS on epithelial integrity and bacterial uptake in the polarized human enterocyte-like cell line Caco-2. *Circ Shock* **40**, 276-88 (1993).
31. Zamora, R. et al. Intestinal and hepatic expression of BNIP3 in necrotizing enterocolitis: regulation by nitric oxide and peroxynitrite. *Am J Physiol Gastrointest Liver Physiol* **289**, G822-30 (2005).
32. Zamora, R. et al. Nitrosative stress in an animal model of necrotizing enterocolitis. *Free Radic Biol Med* **39**, 1428-37 (2005).
33. Crissinger, K.D. Regulation of hemodynamics and oxygenation in developing intestine: insight into the pathogenesis of necrotizing enterocolitis. *Acta Paediatr Suppl* **396**, 8-10 (1994).
34. Ceylan, H. et al. Effects of whole-body hypoxic preconditioning on hypoxia/reoxygenation-induced intestinal injury in newborn rats. *Eur J Pediatr Surg* **15**, 325-32 (2005).
35. Hornef, M.W., Normark, B.H., Vandewalle, A. & Normark, S. Intracellular recognition of lipopolysaccharide by toll-like receptor 4 in intestinal epithelial cells. *J Exp Med* **198**, 1225-35 (2003).
36. Cerqueira, N.F., Hussni, C.A. & Yoshida, W.B. Pathophysiology of mesenteric ischemia/reperfusion: a review. *Acta Cir Bras* **20**, 336-43 (2005).
37. Cavaillon, J.M., Fitting, C., Hauttecoeur, B. & Haeffner-Cavaillon, N. Inhibition by gangliosides of the specific binding of lipopolysaccharide (LPS) to human monocytes prevents LPS-induced interleukin-1 production. *Cell Immunol* **106**, 293-303 (1987).
38. Barman, S.A. Pulmonary vasoreactivity to serotonin during hypoxia is modulated by ATP-sensitive potassium channels. *J Appl Physiol* **83**, 569-74 (1997).
39. Buschard, K., Blomqvist, M., Osterbye, T. & Fredman, P. Involvement of sulfatide in beta cells and type 1 and type 2 diabetes. *Diabetologia* **48**, 1957-62 (2005).
40. Baregamian, N., Song, J., Jeschke, M.G., Evers, B.M. & Chung, D.H. IGF-1 Protects Intestinal Epithelial Cells From Oxidative Stress-Induced Apoptosis. *J Surg Res* (2006).
41. Yang, H.C., Farooqui, A.A. & Horrocks, L.A. Effects of glycosaminoglycans and glycosphingolipids on cytosolic phospholipases A2 from bovine brain. *Biochem J* **299** (Pt 1), 91-5 (1994).
42. Fighera, M.R. et al. Monosialoganglioside increases catalase activity in cerebral cortex of rats. *Free Radic Res* **38**, 495-500 (2004).
43. Moriguchi, N., Yamamoto, S., Isokawa, S., Andou, A. & Miyata, H. Granulocyte functions and changes in ability with age in newborns; Report no. 2: activation of granulocyte functions by cytokines. *Pediatr Int* **48**, 22-8 (2006).
44. Lehrnbecher, T. [Hematopoietic growth factors in prophylaxis and therapy of infections complications in children with neutropenia]. *Klin Padiatr* **213**, 212-38 (2001).
45. Harris, M.C. et al. Cytokine elaboration in critically ill infants with bacterial sepsis, necrotizing entercolitis, or sepsis syndrome: correlation with clinical parameters of inflammation and mortality. *J Pediatr* **147**, 462-8 (2005).

46. Martin, C.R. & Walker, W.A. Intestinal immune defences and the inflammatory response in necrotising enterocolitis. *Semin Fetal Neonatal Med* **11**, 369-77 (2006).
47. Treszl, A., Tulassay, T. & Vasarhelyi, B. Genetic basis for necrotizing enterocolitis--risk factors and their relations to genetic polymorphisms. *Front Biosci* **11**, 570-80 (2006).
48. Nadler, E.P. et al. Expression of inducible nitric oxide synthase and interleukin-12 in experimental necrotizing enterocolitis. *J Surg Res* 92, 71-7 (2000).
49. Pender, S.L. et al. Matrix metalloproteinases in necrotising enterocolitis. *Pediatr Res* **54**, 160-4 (2003).
50. Edelson, M.B., Bagwell, C.E. & Rozycki, H.J. Circulating pro- and counterinflammatory cytokine levels and severity in necrotizing enterocolitis. *Pediatrics* **103**, 766-71 (1999).

CHAPTER 6
THESIS CONCLUSIONS AND FUTURE DIRECTIONS

6.1 THESIS CONCLUSIONS

The hypotheses tested in this thesis were addressed as follows:

Hypothesis 1: Differentiated CaCo-2 cells will have greater amounts of total gangliosides, GD3 and polar/complex gangliosides than undifferentiated CaCo-2 cells thereby resembling the ganglioside composition of human colostrum and neonatal rat intestine. A decrease in the GM3:GD3 ratio will be observed when CaCo-2 cells are differentiated.

A 20 day period of differentiation in culture increased the total amount of gangliosides in CaCo-2 cells (Chapter 3). The ganglioside compositional change that occurred following differentiation included an increase in GD3 and polar complex gangliosides (Chapter 3). Although the GM3:GD3 ratio of CaCo-2 cells decreased following differentiation, the change was not significant (Chapter 3). The decrease in GM3 levels following differentiation was not as drastic as the GM3 increase observed during lactation. This study suggests that differentiated CaCo-2 cells have a ganglioside content and composition that is similar to colostrum and developing rat intestine. Due to the discrepancies in GM3 levels and changes in the GM3:GD3 ratio, further research is needed to evaluate differentiating CaCo-2 cells as a model for studying the physiology and the pathophysiology of neonatal gut.

Hypothesis 2: Based on previous in vitro ganglioside experiments with *Giardia* trophozoites and the lipid bound sialic acid content of human colostrum, the ganglioside concentration range that will demonstrate efficacy without toxicity to CaCo-2 cells will be 4-9 μg/ml of cell culture media.

Human intestine CaCo-2 cell sensitivity experiments with a range of ganglioside GD3 concentrations (0-64 μg/ml) revealed that ganglioside GD3 begins to exhibit

toxicity to enterocytes at concentrations greater than 32 μg/ml (Chapter 4). Therefore, ganglioside GD3 may be safely administered to access nutrient kinetics and distribution in animals and humans at concentrations several times higher than human milk. This study suggests that a stronger therapeutic effect in the absence of toxicity may potentially be achieved when GD3 is administered at concentrations between 9-30 μg/ml.

Hypothesis 3: Based on ganglioside GM1 uptake studies with other cell lines, ganglioside GD3 uptake will be time- and concentration- dependent. GD3 uptake will reach a plateau. Based on sphingomyelin digestion and absorption experiments in rodents, apical GD3 uptake will be greater than 40% efficient. Since drugs are generally best delivered to tissues from the circulation, basolateral GD3 uptake by CaCo-2 cells will be more efficient than apical GD3 uptake.

Amount and percentage ganglioside GD3 uptake from the apical and basolateral side of human intestinal CaCo-2 cells was measured over time and compared. Ganglioside GD3 uptake from the apical and basolateral membrane of human intestine CaCo-2 cells was time-dependent, followed second order kinetics and reached a plateau (Chapter 4). Concentrations of 5 and 10 μg/ml GD3 were administered on the basolateral side of human intestinal CaCo-2 cells and the amount of GD3 taken up was measured. Twice as much GD3 was taken up by enterocytes when double the amount of GD3 was given indicating that basolateral GD3 uptake is concentration-dependent (Chapter 4). Apical and basolateral uptake of ganglioside GD3 was greater than 70% efficient (Chapter 4). Basolateral GD3 uptake was 22% more efficient than apical GD3 uptake (Chapter 4). This study suggests that the brush border membrane and basolateral membrane of enterocytes may contain a carrier that tightly regulates intracellular concentrations of ganglioside GD3. Alternatively, ganglioside GD3 uptake may be passive and dependent on a concentration gradient. Further research is required to test whether ganglioside GD3 uptake is active, passive or carrier-dependent. This study demonstrates that basolateral delivery of GD3 is a more efficient route to target GD3 into the enterocyte than apical delivery of GD3 and that the amount of GD3 taken into

the enterocyte can be increased by administering a higher dose. Implication for ganglioside supplementation to prevent or treat intestinal disorders is suggested.

Hypothesis 4: Route of delivery will influence GD3 fate. Since Dr. Park demonstrated appearance of dietary GD3 in intestinal mucosa lipid rafts and plasma, it is hypothesized that apical GD3 will be retained, metabolized and transferred across the basolateral membrane. Knowing that GD3 functions in modulating cell signaling pathways, it is hypothesized that basolateral GD3 will be retained by cells and metabolized.

The fate of GD3 taken up from the apical and basolateral side of CaCo-2 cells was determined by calculating the retention of GD3 in the cell, the transfer of GD3 across the BBM or BLM and GD3 metabolism. Apical GD3 was primarily metabolized, however, as the amount of GD3 increased inside the cell more GD3 was retained in the cell or transferred across the basolateral membrane (Chapter 4). In contrast to apical GD3, basolateral GD3 was destined for metabolism regardless of the intracellular concentration of GD3 (Chapter 4). This study suggests that the needs of the enterocyte are priority and met by the first portion of GD3 taken up. Excess apical GD3 is transferred across the basolateral membrane to be made available for transport to other tissues while excess basolateral GD3 is metabolized to protect the cell from apoptosis.

Hypothesis 5: Viable, non-inflamed infant bowel may be collected, transported and cultured following intestinal surgery. Cultured infant bowel will undergo necrosis and produce vasoactive and inflammatory signals in response to enterotoxic *E. coli* LPS and the oxygen chelator sodium thioglycollate.

Hematoxylin and eosin staining was used to confirm that infant bowel obtained during intestinal surgery from regions outside the necrotic segment, were healthy with intact villi and no infiltration of immune cells (Chapter 5). Measuring lactate dehydrogenase release as an indicator of cell necrosis, showed that infant bowel remained viable for 24 h and under went necrosis following treatment with LPS or

sodium thioglycollate alone (Chapter 5). Nitric oxide, endothelin-1, serotonin, pro-inflammatory eicosanoids, hydrogen peroxide and pro-inflammatory cytokines were produced by infant bowel following treatment with LPS and sodium thioglycollate (Chapter 5). These results suggest that cultured infant bowel treated with LPS and sodium thioglycollate is a good model for studying the pathogenesis and treatment of necrotizing enterocolitis.

Hypothesis 6: Incubation with ganglioside reduces necrosis of infant bowel during LPS exposure and hypoxia.

Infant bowel necrosis was reduced by gangliosides following LPS treatment (Chapter 5). It was surprising to find that bowel necrosis was not as prominent following hypoxia treatment or exposure to LPS and hypoxia. Since less bowel necrosis was present following hypoxia treatments, ganglioside reduction of necrosis was not significant (Chapter 5). Possible explanations for the reduced ability of hypoxia to trigger necrosis includes a brief period of ischemic pre-conditioning during intestinal surgery or the model represents an earlier time point in disease progression where the transition from apoptosis to necrosis is not yet complete. Alternatively, the absence of true blood flow and gut motility in this ex vivo model may explain the weak response to hypoxia. This study suggests potential use of gangliosides to prevent gut infections during the neonatal period and shows that hypoxia alone may not be the primary trigger for necrotizing enterocolitis. Perhaps ischemia involving reduced blood flow (i.e. oxygen and nutrients) plays a more important role. More research is required to clarify whether hypoxia and ischemia are primary or secondary events in the development of NEC.

Hypothesis 7: Incubation with ganglioside reduces infant bowel production of nitric oxide, endothelin-1 and serotonin during LPS exposure and hypoxia. Moreover, gangliosides will reduce infant bowel production of eicosanoids, hydrogen peroxide and pro-inflammatory cytokines during LPS exposure and hypoxia.

Gangliosides reduced infant bowel production of nitric oxide following LPS exposure and hypoxia and endothelin-1 release following LPS treatment (Chapter 5). The reduction in serotonin release following hypoxia by gangliosides was not significant (Chapter 5). This study suggests that gangliosides exert protective effects on infant bowel by restoring vasoactive mediator balance during LPS exposure and hypoxia. Serotonin may play a role in the pathogenesis of intestinal ischemia but further research is required to clarify the importance of serotonin in the pathogenesis of necrotizing enterocolitis.

Pro-inflammatory mediators produced by the intestine were measured following LPS and hypoxia treatment to determine whether gangliosides modulate infant bowel production of inflammatory signals during LPS exposure and hypoxia. The results show that gangliosides reduce infant bowel production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8), pro-inflammatory eicosanoids (PGE_2, LTB_4) and hydrogen peroxide (Chapter 5) during LPS exposure and hypoxia. Infant bowel generation of IL-10, IL-12 and IFN-γ was below detection limits (Chapter 5). This study suggests that gangliosides exert protective effects on infant bowel by down-regulating pro-inflammatory signals during LPS exposure and hypoxia. It also addresses the need for more sensitive analytical methods to measure trace levels of cytokines in immature bowel.

6.2 Thesis Summary

This thesis demonstrates that ganglioside (1) composition of differentiated CaCo-2 cells somewhat resembles the ganglioside composition present in human colostrum and developing rat intestine; (2) GD3 exhibits no toxicity to enterocytes at levels several times higher than that present in human milk; (3) GD3 uptake from the apical and basolateral side of the enterocyte is fast, efficient, time- and concentration-dependent and may involve a carrier or concentration gradient; (4) GD3 uptake efficiency and fate is influenced by ganglioside structure and route of delivery; (5) GD3 intracellular levels are tightly regulated by the enterocyte; and (6) ganglioside reduces infant bowel necrosis, vasoactive signals and inflammatory signals during LPS exposure and hypoxia.

6.3 Significance of Thesis Research Findings

This is the first research to demonstrate that the ganglioside composition of differentiated CaCo-2 cells is similar to that of human colostrum and developing rat intestine. This finding is significant as it suggests that enzymes involved in ganglioside synthesis, modification and degradation during cancer progression may be similar to those involved in lactation and infant gut development. The structural and functional changes that occur during different stages of colon cancer cell differentiation should be examined further. Since it is ethically difficult to obtain significant amounts of infant intestinal tissue for research, human cancer cell lines such as CaCo-2 may alternatively provide valuable insight into the physiology and pathophysiology of neonatal gut.

The concentration range of ganglioside GD3 that exhibits efficacy without toxicity to the enterocyte was found to be several times higher than the concentration present in human milk. The implication of this finding is that higher concentrations of ganglioside GD3 may be administered to animals or humans to access nutrient kinetics or therapeutic potential without exhibiting toxic side effects.

For the first time, ganglioside GD3 administration to human enterocytes from the apical and basolateral side of the enterocyte was demonstrated to be fast, efficient and dependent on time, concentration and perhaps a carrier. The implication of this important finding is that ganglioside GD3 may be delivered enterally or intravenously to the gut to enhance development or treat disease. Moreover, the present results open a new research area for identification and characterization of enterocyte carriers for glycosphingolipids. Several glycosphingolipid disorders are associated with ganglioside accumulation within cells which may be due in part to defects in ganglioside transport. Accumulating evidence also suggests that sphingolipids compete with other lipids for intestinal uptake. Thus, identifying sphingolipid enterocyte carriers and agents that promote or inhibit transport may have clinical potential in treating a wide range of disorders in lipid metabolism.

This research also demonstrates that ganglioside structure and route of delivery influence ganglioside uptake efficiency and fate. This finding is of prime importance when deciding which ganglioside to administer by which route for different therapeutic purposes. For instance, if the goal was to treat colon cancer, delivering GD3

intravenously may have a superior effect compared to enteral delivery as more GD3 would enter the enterocyte and be metabolized to generate bioactive by-products that kill cancer cells. If the goal was to treat intestinal infection and inflammation, delivering GM1 and GD3 enterally may be the preferred choice as GM1 would remain at the luminal surface while GD3 would exert anti-inflammatory effects throughout the intestinal wall. The uptake of other ganglioside species by human enterocytes should be examined. Further in vivo work is required to understand tissue distribution of individual gangliosides administered by different routes.

Finally, gangliosides were demonstrated to protect infant bowel during LPS exposure and hypoxia by modulating vasoactive and pro-inflammatory signals. A direct implication of this finding is that gangliosides may reduce newborn susceptibility to gut inflammation triggered by infections or ischemic events that are especially common in preterm infants. Preterm birth, infections, ischemia and enteral feeding are established risk factors for the development of necrotizing enterocolitis. Unlike mother's milk, most commercially available infant formulas contain little if any gangliosides. Gangliosides are also absent from total parenteral nutrition. In light of the importance of gangliosides in protecting gut function during infection and ischemia in this infant bowel model, ganglioside supplementation should be considered for infants, especially preterm infants and infants who are unable to breast feed since they are at highest risk for developing necrotizing enterocolitis. From a broader perspective, this research may also be relevant to chronic inflammatory processes of the intestine including Crohn's disease and ulcerative colitis.

6.4 Future Directions

Research in the area of gangliosides is at an early stage and growing with numerous future opportunities to enhance knowledge in this exciting field. Based on an extensive review of the ganglioside literature there are three major areas that require more research attention: mechanism of action, pharmacokinetics/pharmaceutics and broader clinical application. In the final section of this thesis, I will address these issues by providing examples of future follow-up studies that could be done to improve knowledge in these three areas.

6.4.1 Mechanism of Action

It is well established that gangliosides exhibit biological effects through binding of microorganisms and inhibition of protein function via interactions with phosphorylation or calcium signaling pathways[1]. In this thesis, gangliosides were shown to inhibit release of vasoactive mediators including nitric oxide and endothelin-1 (Chapter 5) but the mechanistic detail was not investigated but rather predicted based on previous findings in the literature. Research studies examining the effect of gangliosides on nitric oxide synthesis are controversial. It is apparent that ganglioside structure largely influences the biological actions of gangliosides on nitric oxide synthesis. For instance, GM1 and GT1b inhibit constitutive nitric oxide synthesis while GM3 does not[2,3]. Constitutive nitric oxide synthase produces physiological levels of nitric oxide which promote blood flow and wound healing while exerting anti-thrombotic and anti-inflammatory effects[4,5]. Moreover, GD3 inhibits expression of inducible nitric oxide synthase, the isoform that generates toxic levels of nitric oxide that promote inflammation and apoptosis[6]. A strong mechanistic study that would clarify the effect of gangliosides on nitric oxide levels in the body would separate the effect of individual gangliosides on all three isoforms of nitric oxide synthase. A ganglioside species that specifically inhibits inducible nitric oxide synthase while maintaining or promoting constitutive nitric oxide production would be attractive therapeutically for a wide range of disorders including inflammatory bowel disease, atherosclerosis, ischemia-reperfusion injury and wound healing. Ganglioside GD3 is a potential therapeutic candidate since it has been shown to inhibit inducible nitric oxide synthase and has a similar structure to GM3 which does not inhibit constitutive nitric oxide synthesis.

Another emerging area of therapeutic potential is the identification of compounds that inhibit endothelin-1 production or selectively antagonize endothelin A receptors. Endothelin-1 is a naturally occurring polypeptide which possesses a broad range of activities including vasoconstrictive, pro-inflammatory and pro-fibrotic properties[7]. Endothelin A and B receptors exert similar biological effects with the exception of endothelin receptor B which stimulates production of vasodilatory compounds[7]. In this thesis, gangliosides inhibited endothelin-1 production during infection (Chapter 5). A follow-up study should be done to determine how gangliosides

reduce endothelin-1 synthesis and whether gangliosides possess the ability to antagonize endothelin-1 interaction with different endothelin receptor subtypes. Further investigation of ganglioside effects on endothelin-1 may reveal new therapeutic avenues for gangliosides.

6.4.2 Pharmacokinetics/Pharmaceutics

In this thesis, an in vitro absorption study with ganglioside GD3 was completed and demonstrated potential in delivering gangliosides enterally or intravenously to the intestine and blood (Chapter 4). In vitro systems for uptake of gangliosides across the blood brain barrier or skin barrier should also be investigated. Since gangliosides are naturally present in food and tissues and dietary gangliosides enriched with GD3 have been fed to animals without side effects[8,9], GD3 may be incorporated into enteral products for therapeutic purposes. However, further research is required to provide evidence for safety and efficacy of ganglioside GD3 delivery by other routes including intravenous and transdermal delivery. Moreover, the distribution of gangliosides into plasma lipoproteins and their amphiphilic nature may necessitate research in the area of ganglioside carriers such as liposomes. Ganglioside GD3-containing liposomes have been synthesized[10] and may promote transfer of gangliosides between membranes. Liposomal delivery of nutrients has also been shown to facilitate penetration of lipid bilayers[11]. An advantage of liposomes is that they consist solely of naturally occurring constituents, potentially reducing or eliminating regulatory hurdles that may prevent their clinical application[11]. Perhaps GD3-containing liposomes may promote transfer and uptake of ganglioside GD3 by tissues. A follow-up in vivo study should be completed to examine the safety and tissue distribution of liposomal GD3 delivered intravenously.

6.4.3 Broader Clinical Applications

The results of this thesis suggest clinical potential for ganglioside supplementation in infant formula to promote gut development and prevent infections and necrotizing enterocolitis. Along with in vivo ganglioside research findings[8], this study has led to initiation of a pilot clinical trial on the safety and efficacy of dietary

gangliosides in inflammatory bowel disease. Thus, this section will focus on other broad clinical applications.

6.4.3.1 Atherosclerosis and Prevention of Myocardial Infarction and Stroke

Preliminary research on the role of gangliosides in cardiovascular disease and stroke has begun and is controversial. Most studies have focused on the potential of gangliosides, especially GM1, in reducing ischemic heart and brain injury[3,12]. More recently, ganglioside GM3 has been demonstrated to be elevated in atherosclerotic lesions and involved in foam cell formation[13] while GD3 has been shown to inhibit vascular smooth muscle responses[14]. Effects of ganglioside GD3 on the pathogenesis of cardiovascular disease and stroke deserve more research attention. Dietary GD3 may potentially reduce levels of pro-atherogenic GM3 in atherosclerotic lesions. The vasoactive and inflammatory mediator results of this thesis suggest that GD3 may normalize nitric oxide and endothelin levels, promoting blood flow and anti-thrombotic effects while suppressing inflammation and fibrosis. Moreover, the GD3 absorption results suggest that GD3 may be more bioavailable and of greater therapeutic potential than GM1. If GD3 is taken up by a cholesterol transporter, it may be able to compete with and inhibit cholesterol absorption thereby reducing the formation of lipid rich atherosclerotic plaques.

6.4.3.2 Prevention of Ischemic Injury in Organ Transplantation

During and after transplantation, the transplanted organ experiences a variety of insults (for example during donor procurement, organ retrieval, cold storage, transplantation, rejection episodes, administration of immunosuppressive drugs or through non-immunological factors such as hypertension and hyperlipidemia) that result in functional impairment and structural damage, followed by an inflammatory response as part of the repair mechanism[15]. Ischemia-reperfusion injury is the main cause of poor function and significantly reduces tissue regeneration[16,17]. The inflammatory response consists of the activation and recruitment of immune cells that infiltrate the graft and synthesize mediators along with local endothelial and graft parenchymal cells[15]. These mediators include pro-inflammatory cytokines, vasoactive substances (endothelin-1 and

nitric oxide), pro-fibrotic substances (endothelin-1) and growth factors (TGF-β)[15]. Minimizing the adverse effects of ischemia-reperfusion injury could increase the number of both suitable transplantation grafts and of patients who successfully recover from organ transplant[16,17]. Gangliosides may reduce inducible nitric oxide synthase expression during rejection episodes and maintain constitutive nitric oxide production. Constitutive nitric oxide promotes blood flow and healing while reversing immunosuppressive drug toxicity[15]. Gangliosides may also reduce inflammation and fibrosis by inhibiting production of pro-inflammatory cytokines and endothelin-1. Further mechanistic investigation on ganglioside effects is required to confirm potential application in preventing ischemic injury present in organ transplantation.

6.4.3.3 Systemic Sclerosis

Systemic sclerosis is a chronic connective tissue disease of unknown etiology characterized by progressive fibrosis of the skin and internal organs[18]. Vascular impairment, immunological disturbances and inflammation are considered important elements of the pathogenic process in systemic sclerosis[18]. The ability of gangliosides to restore vasoactive mediator balance and reduce inflammation makes gangliosides an attractive therapeutic agent for systemic sclerosis. Endothelin antagonists and agents that restore nitric oxide balance have demonstrated beneficial effects in systemic sclerosis clinical trials[7,18].

6.4.3.4 Shock in Trauma, Burn or Critically Ill Patients

Factors derived from the intestine are important contributors to shock in critically ill and injured patients[19]. Trauma, burn and critically ill patients especially those developing multiple organ dysfunction syndrome frequently acquire life-threatening bacteremias with enteric organisms[20]. One factor that most stress and injury models of bacteria translocation share is reduced splanchnic blood flow, leading to ischemia-reperfusion mediated gut injury[19]. Thermal injury and major burns are associated with mesenteric vasoconstriction and result in increased bacterial translocation and endotoxin absorption from the gut[21]. Intestinal permeability is also increased in patients with major thermal injuries shortly after the burn as well as during

episodes of infection[22,23]. Moreover, burn patients have an alarming high incidence of ischemic intestinal complications, as shown by an autopsy study of 161 burn patients[24]. A recent study in rats demonstrated that nitric oxide and endothelin-1 are elevated in the plasma following thermal injury, originated from the burn tissue and that removal of the injured tissue had beneficial effects on blood flow[25]. Thus, gangliosides may potentially be used in trauma, burn and critically ill patients to prevent infections, reduce ischemic gut damage and inflammation and promote blood flow and healing.

6.5 References

1. Ledeen, R.W. & Wu, G. Ganglioside function in calcium homeostasis and signaling. *Neurochem Res* 27, 637-47 (2002).
2. Dawson, T.M., Hung, K., Dawson, V.L., Steiner, J.P. & Snyder, S.H. Neuroprotective effects of gangliosides may involve inhibition of nitric oxide synthase. *Ann Neurol* 37, 115-8 (1995).
3. Tseng, E.E. et al. Monosialoganglioside GM1 inhibits neurotoxicity after hypothermic circulatory arrest. *Surgery* 124, 298-306 (1998).
4. Hackam, D.J., Upperman, J.S., Grishin, A. & Ford, H.R. Disordered enterocyte signaling and intestinal barrier dysfunction in the pathogenesis of necrotizing enterocolitis. *Semin Pediatr Surg* 14, 49-57 (2005).
5. Upperman, J.S. et al. Mechanisms of nitric oxide-mediated intestinal barrier failure in necrotizing enterocolitis. *Semin Pediatr Surg* 14, 159-66 (2005).
6. Zamora, S.A. et al. Plasma L-arginine concentrations in premature infants with necrotizing enterocolitis. *J Pediatr* 131, 226-32 (1997).
7. Kowal-Bielecka, O. & Sierakowski, S. Endothelin receptor antagonism--new perspectives in the treatment of systemic sclerosis. *Rocz Akad Med Bialymst* 50 Suppl 1, 291-3 (2005).
8. Park, E.J. et al. Dietary ganglioside decreases cholesterol content, caveolin expression and inflammatory mediators in rat intestinal microdomains. *Glycobiology* 15, 935-42 (2005).
9. Vesper, H. et al. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J Nutr* 129, 1239-50 (1999).
10. Masso, O., Alino, S.F., Lejarreta, M., Blasco, F. & Piulats, J. Specific serological response by active immunization with GD3-bearing liposomes. *J Pharmacol Exp Ther* 278, 1114-20 (1996).
11. Taylor, T.M., Davidson, P.M., Bruce, B.D. & Weiss, J. Liposomal nanocapsules in food science and agriculture. *Crit Rev Food Sci Nutr* 45, 587-605 (2005).
12. Dolara, P., Lodovici, M., Fazi, M., Lasagni, L. & Sturlini, S. Protective effect of gangliosides on myocardial hypoxic damage in the rat. *Eur J Pharmacol* 198, 43-9 (1991).
13. Bobryshev, Y.V. et al. Expression of GM3 synthase in human atherosclerotic lesions. *Atherosclerosis* 184, 63-71 (2006).

14. Moon, S.K., Kim, H.M., Lee, Y.C. & Kim, C.H. Disialoganglioside (GD3) synthase gene expression suppresses vascular smooth muscle cell responses via the inhibition of ERK1/2 phosphorylation, cell cycle progression, and matrix metalloproteinase-9 expression. *J Biol Chem* 279, 33063-70 (2004).
15. Demetriou, D., Wenter, C. & Watschinger, B. Vasoactive substances in renal transplantation. *Curr Opin Urol* 10, 63-9 (2000).
16. Casillas-Ramirez, A. et al. [Ischemia-reperfusion syndrome associated with liver transplantation: an update]. *Gastroenterol Hepatol* 29, 306-13 (2006).
17. Casillas-Ramirez, A., Mosbah, I.B., Ramalho, F., Rosello-Catafau, J. & Peralta, C. Past and future approaches to ischemia-reperfusion lesion associated with liver transplantation. *Life Sci* 79, 1881-94 (2006).
18. Kowal-Bielecka, O. Targeting vascular disease in systemic sclerosis. *Endocr Metab Immune Disord Drug Targets* 6, 401-7 (2006).
19. Magnotti, L.J. & Deitch, E.A. Burns, bacterial translocation, gut barrier function, and failure. *J Burn Care Rehabil* 26, 383-91 (2005).
20. Deitch, E.A. Multiple organ failure. Pathophysiology and potential future therapy. *Ann Surg* 216, 117-34 (1992).
21. Maejima, K., Deitch, E.A. & Berg, R.D. Bacterial translocation from the gastrointestinal tracts of rats receiving thermal injury. *Infect Immun* 43, 6-10 (1984).
22. Deitch, E.A., Morrison, J., Berg, R. & Specian, R.D. Effect of hemorrhagic shock on bacterial translocation, intestinal morphology, and intestinal permeability in conventional and antibiotic-decontaminated rats. *Crit Care Med* 18, 529-36 (1990).
23. Ziegler, T.R., Smith, R.J., O'Dwyer, S.T., Demling, R.H. & Wilmore, D.W. Increased intestinal permeability associated with infection in burn patients. *Arch Surg* 123, 1313-9 (1988).
24. Desai, M.H., Herndon, D.N., Rutan, R.L., Abston, S. & Linares, H.A. Ischemic intestinal complications in patients with burns. *Surg Gynecol Obstet* 172, 257-61 (1991).
25. Chen, X., Soejima, K., Nozaki, M., Tanabe, Y. & Sakurai, H. Effect of early wound excision on changes in plasma nitric oxide and endothelin-1 level after burn injury: an experimental study in rats. *Burns* 30, 793-7 (2004).